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PROFESSOR WILLIAM RIDDET, C.B.E.

PROFESSOR WILLIAM RIDDET

Readers will have heard with much regret of the death on 30 December 1958 of Professor William Riddet at the age of 62.

Riddet was born, and remained, a Scot with all of those excellent attributes we expect in the best of his countrymen. He was associated with the *Journal of Dairy Research* from the start, his name appearing on Volume 1, Number 1, November 1929, as overseas correspondent in New Zealand.

His student career at the West of Scotland College of Agriculture and Glasgow University was brilliant. After a short period as lecturer at the West of Scotland College he accepted in 1925 the newly created Professorship of Agriculture at Auckland University College, New Zealand. Before leaving England he spent some time here at the National Institute for Research in Dairying, making contacts which were never broken. I well remember the impression he made on me. Although he seemed very young for a Professorship I was in no doubt as to his capacity. Having quickly impressed the authorities with his success, in collaboration with Sir Geoffrey Peren, in the herculean task of founding the Massey Agricultural College which also accommodated the Dairy Research Institute (New Zealand Department of Scientific and Industrial Research), he became almost as of right Professor of Dairying at the College and Director of the Institute. Between the N.I.R.D. and the N.Z. Research Institute there has always been a strong, indeed a brotherly, bond. Witness only the fact that one of Riddet's last imaginative pieces of work was the dispatch to our Bacteriology department of a team of workers, as much for the mutual picking of brains as for the search for a solution of a particular problem. Riddet had long wished for this kind of exchange of workers and he lived to see its achievement.

He quickly showed that he was a first-class administrator and maintained his capacity to the very last. When I saw him some three years ago, he had already fallen a victim to the leukaemia which in the end proved fatal. He was under no delusions about his condition, but he was looking and planning ahead as he always did.

It was Sir Ben Lockspeiser who said: 'Let me therefore conclude by underlining the importance of good administration, but by reminding you also that administration in science will not, of itself, produce a single new idea and without new ideas science would cease to exist.' There was no question of lack of new ideas with Riddet. He was most exuberant—so much so that without the enthusiastic co-operation of his colleagues he could never have brought a fraction of his plans to fruition. But to fruition many of them came and the excellence of the work of his Institute, at the hands of Dr Whitehead and Dr McDowall, to mention only two, is universally acknowledged and acclaimed.

He was as thorough as a teacher as he was as an administrator. But he also endeared himself to his friends by his enthusiasm in the smaller things—for example, as a trustee of St Peter's School, Cambridge, N.Z., he made a hobby of managing their dairy farm for many years.

His reputation being made, he was widely honoured, and in 1954 was created a C.B.E.

The memory of his life and works will endure.

A. T. R. Mattick

National Institute for Research in Dairying, Shinfield, Reading

VOLUME 26, NO. 2 JUNE 1959

MAKING CHEDDAR CHEESE ON A SMALL SCALE UNDER CONTROLLED BACTERIOLOGICAL CONDITIONS

BY L. A. MABBITT, HELEN R. CHAPMAN AND M. ELISABETH SHARPE

National Institute for Research in Dairying, Shinfield, Reading

(*Received* 15 *October* 1958)

(With 3 Figures)

The importance of streptococci as producers of acid in the Cheddar cheese process is unquestioned. It is, however, often assumed that they and also the adventitious organisms, e.g. lactobacilli, micrococci, and coliforms, are also responsible for important physical and chemical changes during ripening. This assumption has initiated many studies of the microflora of cheese (1,2) and inoculation experiments designed to improve cheese flavour (3, 4). Nevertheless, the importance of micro-organisms in ripening is still uncertain and can be determined only when the microflora of cheese can be controlled, for example by excluding the microflora of the dairy and the cheesemakers from the cheese vat. The apparatus described below has been designed for this purpose.

Organisms present in the milk supply may, of course, grow in the cheese. Heat sterilization of milk for cheese-making unfortunately results in a weak curd, but suitable pasteurizing conditions may be chosen which destroy lactobacilli and most of the other milk organisms which might grow in Cheddarc heese, without unduly affecting the cheesemaking (5). The initial objective has, therefore, been limited to the manufacture of Cheddar cheese containing no lactobacilli so that their role in cheese ripening can be assessed.

APPARATUS AND METHODS

Heat treatment of the milk. An A.P.V. HX-type heat exchanger was employed (6). Sterile milk for use in testing the efficiency of the special vat was prepared at 135° C. with a holding time of 2–4 sec. Milk for cheese-making free from lactobacilli was obtained by pasteurizing at 160° F. for 17 sec. (5).

Cheese vats. Two 50 gal. rectangular vats fitted with reciprocating stirrers were connected to the heat exchanger by standard pipe-line (Fig. 1). Water from a common reservoir *e* could be heated by steam injection and pumped through the jacket of each vat. This enabled the contents of both vats to be controlled at identical temperatures during cheese-making.

Special vat cover. In order to exclude micro-organisms from one of the vats (vat A) a special cover was fitted (Fig. 2), consisting of a stainless steel frame into which 2 large windows of heat-tempered glass were inserted and sealed with rubber gaskets. Two stainless steel flanges fitted into each window were similarly sealed and rubber gauntlets,* attached to the flanges and bound with cotton-wool at the joint, were used for all the cheese-making operations in the vat, thus avoiding contact between the operator and the

* Supplied by All British Workers, Northampton.

interior of the vat. The ends of the cover were of stainless steel sheet suitably cut away in the central area to enable them to sit accurately into flanged Tufnol end-seals which fitted closely to the stirrer shaft (Fig. 2.)

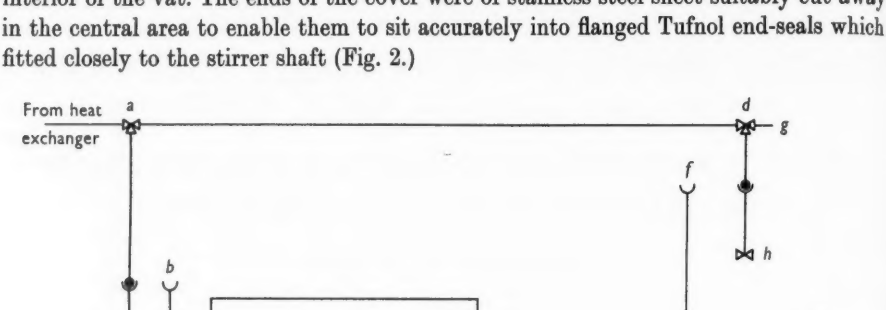

Fig. 1. Diagram showing arrangement of milk pipelines serving the aseptic vat A and control vat B. *a, c, d, h,* cocks; *b, f,* inlet pipes; *e,* water reservoir controlling temperature of both vats; *g,* thermometer.

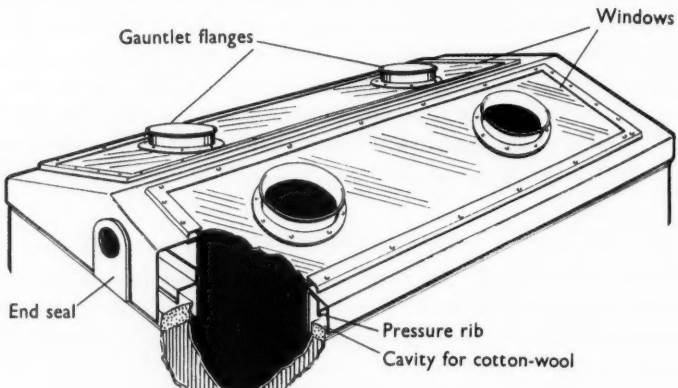

Fig. 2. Cover of aseptic vat.

An inlet pipe (Fig. 1, *b*) soldered into one end of the cover was used for the aseptic introduction of milk, starter and rennet. The entrance could be closed with a sterile blank or connected to the milk supply line as required.

The joint between the cover and the vat was made with cotton-wool retained in the inverted trough formed by the edges of the steel frame of the cover (Fig. 2). The inner edge also served to direct condensate away from the cotton. A metal rib fixed centrally in the trough and running parallel to it compressed the cotton-wool along the top edges of the vat to provide an adequate seal.

Equipment in the vat. The minimum amount of equipment needed for cheese-making was suspended from the stirrer shaft (Fig 1). Two cheese knives of special length were held by wire hooks, and a basket which was tied on contained a thermometer, a curd knife, screw-capped sample bottles and two jars. The latter held in a dry condition a weighed quantity of sterile salt and a sterile bag made of cheese cloth into which the milled curd was packed at the end of the making process.

Sampling and bacteriological testing. Samples of cheese were taken during the ripening period by using a sterile borer, three cores from the same cheese being combined to prepare an homogenate (5). The homgenates of early samples (1 week) were examined for coliform organisms by culturing in McConkey broth. All other samples were plated on acetate medium selective for lactobacilli (7) and on yeast glucose agar and nutrient agar as non-selective media. Colonies were picked from the media for further identification; lactobacilli, micrococci and spore formers by microscopic and cultural tests and streptococci by microscopic appearance and 'phage typing.'

Samples (20 ml.) taken with the gloves from the milk of vat A at the start of cheese-making were plated on acetate medium when the process was completed. Other samples of curd and whey which were taken in a similar manner and also 500 ml. samples of milk from the supply line were enriched for 1 month at 30° C. before plating on the same medium.

TECHNIQUE OF CHEESE-MAKING IN THE SPECIAL VAT

The process for making cheese in the special vat has been frequently modified in the light of experience. The best technique found, which is described below, was substantially in use as from and including cheese no. 3.

Sterilization of milk line and vat assembly. Because of the size of the vat autoclaving was not practicable. The procedure finally adopted was designed to combine a maximum effect on heat-tolerant organisms with a minimum effect on the rubber of the gauntlets.

With the outlets restricted at *c* and *h* (Fig. 1) the whole of the milk pipe-line through the heat exchanger was steamed for ½ hr. (temperature at thermometer *g* 214° F.). The pipe line was then connected to vat A at *b*, the inlet in the special cover, and with the glove holes sealed with paper the steaming was repeated. When cool the cover was removed from the vat, inverted, and the trough packed evenly with cotton wool. Also, the cheese-making equipment (previously autoclaved in a wrapped condition) was unwrapped and suspended from the stirrer shaft of the vat by an operator wearing sterile gloves. The cover was then lowered back into position and the assembly steamed as before and left overnight. Before cheese-making began the following morning sterile gauntlets (autoclaved for 10 min. at 10 lb./in.^{2}) were removed from their wrapping and fitted with aseptic precautions to the glove ports. The complete assembly was then subjected to a final steaming. The steam pressure inside the vat was sufficient to inflate the gloves, and to reveal any weak points in the assembly.

The efficiency of sterilization was assessed by running 15 gal. of sterile milk into the vat from the heat exchanger and collecting duplicate 20 ml. samples using the gloves, (i) as the milk entered the vat, (ii) after stirring for 5 min. and (iii) after stirring overnight in the vat at room temperature. No bacteria could be isolated from samples (i) and (ii), but sample (ii) after enrichment by incubating for 5 days at 30° C. was found to contain about 150 sporeformers/ml. They probably originated from the surface of the vat and had

survived the heat treatment. In a second test, after a thorough scrubbing of the vat before steaming, no bacteria could be isolated even from the enriched samples. It was concluded that the vat cover effectively prevented bacteria gaining access to the vat from the outer environment. Also, any sporing organisms which might survive the heat treatment were unlikely to be of importance as they are known to be incapable of growth in Cheddar cheese.

Vat B received no special treatment. It was washed and steamed before each cheese-making.

Filling the vats with milk. The pasteurized milk was run first into the non-sterile vat B via a sterile inset pipe *f* which was fitted immediately before milk flow began. When about 30 gal. had been delivered two 500 ml. samples were taken aseptically and the milk supply was then switched to the sterile vat A. Large samples reasonably representative of the milk in vat A were thus obtained. In addition, smaller samples of the milk coming into vat A were taken using the rubber gloves. When 30 gal. of milk had been delivered into the vat the milk line at *b* was disconnected and the entry port closed with the sterile blank.

Cheese making. In vat B the cheese was made in the usual way and only the special techniques used in the aseptic vat A will be mentioned.

The same amounts of starter and rennet were added to each vat and as the temperature in each was the same at all times during cheese-making it was expected that the acidities in each vat would follow each other very closely. This was found to be the case in a number of preliminary trials (Table 1). Thus the acidities of the milk and whey in vat B were used to control the cheese-making in both vats and the removal of samples from the aseptic vat A was avoided.

Table 1. *Typical figures for* pH *and temperature of the milk, whey and curd in vats A and B*

Time min.	Operation	Temperature (° F.)		pH	
		Vat A	Vat B	Vat A	Vat B
0	Milk in vats	84	84	6·78	6·78
22	Starter added	86	86	.	.
59	Rennet added	86	86	6·69	6·63
101	Coagulum cut	86	86	6·64	6·60
121	Heat on	86	86	6·51	6·52
159	Heat off	98	98	6·20	6·21
236	Curd settled	98	98	.	.
254	Whey off	97·5	97·6	.	.
264	First turn	94	94	5·50	5·60
339	Curd milled	83	82	5·30	5·28
359	Cheese in press	.	.	5·20	5·20
After 24 hr. pressing		.	.	5·24	5·19

The starter, a culture of *Streptococcus lactis*, was prepared in autoclaved skim-milk in one-pint, screw-capped bottles (4 per vat) with necks of a size to fit snugly into the entry port of vat A. Aseptic introduction of the starter was effected by removing the bottle cap and placing the flamed bottle neck into the flamed entry port. The mechanical stirrer was then started and 30 min. later seitz-filtered rennet was introduced in a similar manner.

Cutting the curd in the enclosed vat with the stirrer in position and with the equipment suspended from the stirrer shaft was a difficult operation. It was best carried out by two operators, one working at each side of the vat.

At whey run-off a considerable volume of air was sucked into the vat. To avoid undue strain on the cotton-wool packing, the flow rate of the whey through the vat cock was restricted. The curd was cut into blocks with the curd knife and cheddared in the usual way. During this period samples of whey were run off through the vat cock to check that the rate of acid production was similar in each vat.

After cheddaring, the curd was pulled, by gloved hands, into pieces about the size of milled curd, salted and packed into the sterile cheese-cloth bag. At this point, with the cheese-maker's hands still in the gloves, the vat cover was raised momentarily for the first time. This enabled a sterile cheese-mould and followers to be introduced into the vat and subsequently allowed the bag of curd to be packed into the mould with little risk of contamination.

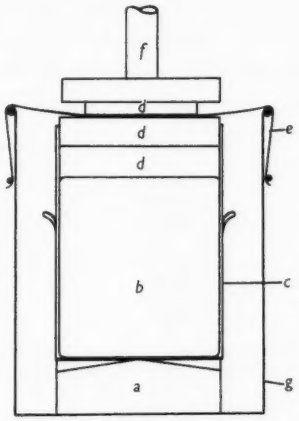

Fig. 3. Diagram showing the pressing of 'aseptically' made cheese in a sterile dustbin. *a*, channelled wood block; *b*, cheese, *c*, cheese mould; *d*, cheese followers; *e*, rubber sheet; *f*, ram of press; *g*, dustbin.

Pressing the curd. The vat cover was now completely removed and the cheese mould containing the curd lifted from the vat with sterile gloves and quickly placed on to a channelled wood block in a sterile dustbin (Fig. 3). This was then covered with a sterile rubber sheet which was bound around the bin with string and through which pressure was applied to the cheese. After 12 hr. in press, the pressure was released and the cheese trimmed with a sterile knife and bathed with sterile water at 150° F. It was then pressed for a further 24 hr. and transferred aseptically to a second sterile bin containing dehydrated silica gel, where it was left overnight. The surface of the cheese was then sufficiently dry to allow an autoclaved liquid plastic (Plasticoat) to be applied with a sterile paint brush to provide a protective surface easily bored when samples were required.

The cheese from vat B was similarly coated and both cheeses were ripened together in the usual way without special precautions.

RESULTS AND DISCUSSION

Six experiments have been completed using the two vats. No lactobacilli were isolated from the enriched milk samples or from any sample taken from aseptic vat A during cheese-making. The results obtained from the cheeses made under the 'aseptic' and normal conditions during the ripening process are shown in Tables 2 and 3. In exps. 1

and 2 the rubber gloves were found to have weakened at a vulcanized joint between cuff and sleeve and several small leaks were detected. These defects were prevented in Exps. 3–6 by strengthening the joint with a light aluminium ring. Thus the failure to prevent lactobacilli from growing in the 'aseptic' cheese 1 (Table 2) is adequately explained. No similar explanation can be offered for contamination which occurred in Exps. 3 and 6. In Exp. 3 large numbers of pediococci which grew well on the acetate agar made it impossible to determine whether lactobacilli were present or not. They have been noted

Table 2. *Numbers of lactobacilli found in 'aseptic' cheeses (series A) and control cheeses (series B) during ripening*

Cheese no.	Age of cheese at sampling			
	1 month	3 months	4½ months	6 months
	Viable counts × 10^{-4} of lactobacilli/g. cheese, on acetate agar			
1A	<0·0005	1·4	.	.
1B	150	9·5	.	.
2A	<0·0005	<0·0005	<0·0005	<0·0005
2B	500	270	1140	218
3A	[700]	[350]	.	.
3B	1090	2050	.	.
4A	<0·0005	<0·0005	<0·0005	<0·0005
4B	120	25	3000	730
5A	<0·0005	<0·0005	<0·0005	.
5B	1470	3100	0·208*	.
6A	<0·0005	0·203	.	.
6B	2	4300	.	.

[ ] Counts of pediococci present on acetate agar.
* Count of lactobacilli + pediococci which were also present.

previously in Cheddar cheese (10) and may have reached abnormally high numbers in this instance in the absence of a competing lactobacillus flora. It is uncertain whether they were originally in the milk supply and withstood pasteurization or whether a breakdown in efficiency of the apparatus occurred.

Other 'aseptic' cheeses (nos. 2, 4 and 5) were found to contain only *Str. lactis* (shown by 'phage typing' to be identical with the starter used) and a few spore formers (Table 3) which were doubtless derived from the milk. Lactobacilli were absent from the duplicate 10^{-1} dilution samples examined, i.e. no lactobacilli were found in 0·2 g. cheese.

In contrast, the cheeses made in the uncovered vat B were found to contain normal numbers of lactobacilli, as well as starter streptococci, sporeformers and sometimes micrococci. In some instances the lactobacilli in these cheeses were so numerous that no streptococci could be isolated on non-selective media (e.g. cheese 2B at 4½ months).

The difficulties of assessing flavour and the few successful experiments which have so far been carried out make it impossible to draw valid conclusions about the effect of the cheese microflora on cheese ripening. It is intended that this aspect will be the subject of a later paper.

The microflora of cheese might be partially controlled in various ways. Cheese-making with milk treated with hydrogen peroxide has been previously tried (8). In this process SH groups and some growth factors are destroyed, the character of the cheese curd may be affected (9) and interpretation of results made difficult. In addition, destruction of the peroxide before cheese-making begins is essential in order to permit the starter organisms

Table 3. *Bacteria isolated from 'aseptic' cheeses (series A) and control cheeses (series B) during ripening*

Cheese no.	Age of cheese at sampling																					
	1 month				3 months				4½ months				6 months									
	Lb.	Str.	Micro.	Ped.	SpF.	Lb.	Str.	Micro.	Ped.	SpF.	Lb.	Str.	Micro.	Ped.	SpF.	Lb.	Str.	Micro.	Ped.	SpF.	Lb.	Str.	Micro.	Ped.	SpF.
1A	—*	.	.	.	.	+	+	−	−	−	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
1B	+*	.	.	−	−	+	−	−	−	−	−	+	−	−	+	−	+	−	−	−	+	+	−	−	+
2A	−	+	+	−	−	+	+	−	−	+	+	−	−	−	+	+	+	+	−	−	+	+	−	−	+
2B	+	+	−	−	−	+	+	−	−	−	+	−	+	−	+	+	+	−	+	−	−	.	.	.	.
3A	−	−	−	+	+	−	−	+	+	−	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
3B	+	+	−	−	+	+	−	−	−	−	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
4A	−	+	+	−	−	+	+	−	−	+	−	+	+	−	+	−	+	−	+	−	+	+	+	−	+
4B	+	+	+	−	−	+	+	+	−	+	+	−	−	−	+	+	+	−	−	−	+	.	.	.	.
5A	−	+	+	−	+	−	+	−	−	+	−	+	+	−	+	+	+	+	−	−	+	.	.	.	.
5B	+	+	+	−	+	+	+	−	−	+	+	+	−	+	+	.	.	.	.	.	.	.	.	.	.
6A	−	+	+	−	−	+	−	−	−	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
6B	+	+	−	−	−	+	−	−	−	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Lb. = labtobacilli; Str. = starter streptococcus 712; Micro. = micrococci; Ped. = pediococci; SpF. = aerobic spore-former.
* Cheese sample plated only on selective medium for lactobacilli.

to multiply. Thus the organisms which find their way into the vat during cheesemaking may subsequently grow in the cheese.

Partial control of the microflora by the addition of antibiotics, e.g. nisin, to cheese is also possible (10). It involves a difficulty that for investigational work a number of antibiotics selective against different species or strains would be required, and in addition the antibiotic must remain stable throughout the ripening period.

Clearly a complete control of the microflora by making cheese under aseptic conditions from sterile milk would be the ideal method. It would enable the effect on cheese quality of different strains or species of microorganisms to be assessed and should be applicable to all types of cheese. It would also allow control of the microflora in any investigation of the cheese-making process.

The most persistent difficulty encountered during the work reported above has been the failure of the rubber gauntlets of appropriate length and available in Britain to withstand the operating conditions. In addition, the diversity of operations involved in cheesemaking introduces many possible sources of contamination. From this viewpoint the results obtained with the 'aseptic vat' are satisfactory. Exploitation of the technique should make possible the successful investigation of many problems concerning the microbiology of cheese and its relationship to cheese ripening.

SUMMARY

A cover for a 40 gal. cheese vat has been specially designed to allow cheese to be made under conditions which prevent the entry of micro-organisms into the vat from the dairy environment. The technique employed is described. Using milk free from lactobacilli it has been possible to make Cheddar cheese in which no lactobacilli grew during the ripening period. An assessment of the role of these and other micro-organisms in cheese ripening should now be possible.

The technique should also be of help in other cheese investigations where control of the microflora is desired.

The authors gratefully acknowledge the criticism and advice of Dr N. J. Berridge, and useful suggestions made by Dr Jill Naylor. They are especially indebted to Mr C. Machin for help in designing the cover, to Dr K. D. Perry for help in some of the bacteriological work, and to Miss A. J. W. Harrison and Miss J. Butler for technical assistance.

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STEAM DISTILLATION OF TAINTS FROM CREAM

VIII. EFFECT OF FAT CONTENT OF DAIRY PRODUCT ON DEODORIZATION PROCESS

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(With 3 Figures)

The vapour/liquid equilibrium coefficient of a tainting substance in a dairy liquid containing butterfat may be markedly different from the coefficient for the substance in water or lactose solution, and it has been shown (1) that the extent of difference is directly related to the solubility distribution coefficient for the substance as between butterfat and skim-milk.

In commercial practice dairy liquids of a wide range of fat contents are subjected to steam distillation. (*a*) In some countries it is found to be an advantage to deodorize market milk, e.g. for removal of wild onion or wild garlic flavours (2,3). The milk may be of fat content as low as 3·0–3·5%. (*b*) Cream received in very acid conditions, e.g. centralizer cream in some areas of United States, requires some dilution with water before it can be satisfactorily neutralized and handled through the processing equipment. And cream which is to be ripened with starter before churning is usually separated to a fat content of 25–30%, because of the difficulty of pumping, pasteurizing and churning a high fat acid cream. Even in factories handling sweet cream it is the practice of some operators to dilute segregated poor quality cream before processing, with the object of obtaining better deodorization. (The impression that deodorization is improved by dilution of the cream is erroneous. As will be shown later, the most efficient removal of taint is obtained with high fat content cream. Dilution can cause a reduction in the final taint content of the butter (i) if the cream and steam flows after dilution are kept the same as before dilution, and (ii) because of the effect on the partition equilibrium at churning). (*c*) In the Gold'n Flow continuous buttermaking process the butterfat/cream-plasma mixture treated in the Vacreator vacuum-pasteurizer *en route* from the destabilizer to the standardizing vats contains 80–95% of butterfat (4).

The present paper is a study of the combined influence of (i) fat content of the product subjected to steam distillation and (ii) butterfat/skim-milk distribution ratio of the tainting substance on the effectiveness of the deodorization treatment obtained.

Effect of fat content of cream on vapour/liquid equilibrium coefficient for substances of different butterfat/skim-milk distribution coefficients, for any one overall concentration of tainting substance in cream

As shown in a previous paper (1) the vapour/liquid equilibrium coefficient (m_c) for a substance in cream of percentage fat content F is given by the formula

$$m_c = m_s \times \frac{100}{100 - F(1-k)}, \qquad \text{(I)}$$

where m_s = the vapour/liquid equilibrium coefficient for the substance in skim-milk; and k = the butterfat/skim-milk distribution coefficient at 100° C. By use of this formula the effect of fat content of cream on the equilibrium coefficient for substances of different butterfat/skim-milk distribution coefficients can be computed. In Fig. 1 the results of such a computation are presented as percentages of the equilibrium coefficient corresponding to a butterfat/skim-milk distribution coefficient of 1.

From the curves in Fig. 1 it is clear that when the substance has a low distribution coefficient, i.e. when it is relatively more soluble in skim-milk than in butterfat, there is a marked advantage in separating to a cream of high fat content. For a substance with a butterfat/skim-milk distribution ratio of 1:100 the vapour/liquid equilibrium coefficient for a 40% cream is 166% of the coefficient for a substance of equal steam volatility, but with a butterfat/skim-milk distribution ratio of 1:1. The corresponding value for an 80% cream is 480%. When a tainting substance has a butterfat/skim-milk distribution ratio higher than 1:1 the vapour/liquid equilibrium coefficient decreases with rise in fat content of the cream. The change is rapid with increase in fat content for low-test creams, and more gradual for high-test creams. Butterfat/skim-milk distribution ratios of 10:1 and 100:1 reduce the vapour/liquid equilibrium coefficient for a 40% cream to 21 and 2·4%, respectively, of what the coefficient would have been if the distribution ratio had been 1:1. The corresponding figures for an 80% cream are 12 and 1·2% respectively. (Compare experimental results for reference substances (1).)

Effect of fat content of cream separated from milk on concentration of tainting substance in cream

In milk a tainting substance is distributed between the fat and the skim-milk portions in accordance with the butterfat/skim-milk distribution coefficient at the temperature obtaining. When the milk is separated, the concentrations of the tainting substance in the fat and the cream plasma are the same as those in the fat and milk plasma, if the milk is separated without preheating. The content of tainting substance in the cream from any one milk then will vary with (*a*) the percentage fat content of the cream separated, and (*b*) the butterfat/skim-milk solubility distribution ratio for the substance at the temperature of separation. The formula connecting percentage fat contents of milk and cream, tainting substance contents of milk and cream, and butterfat/skim-milk distribution coefficient may be derived as follows:

Let C_m = Overall concentration of tainting substance in milk to be separated (μg./g.).
C_c = Overall concentration of tainting substance in the cream separated.
C_f = Concentration of tainting substance in the butterfat (μg./g.).

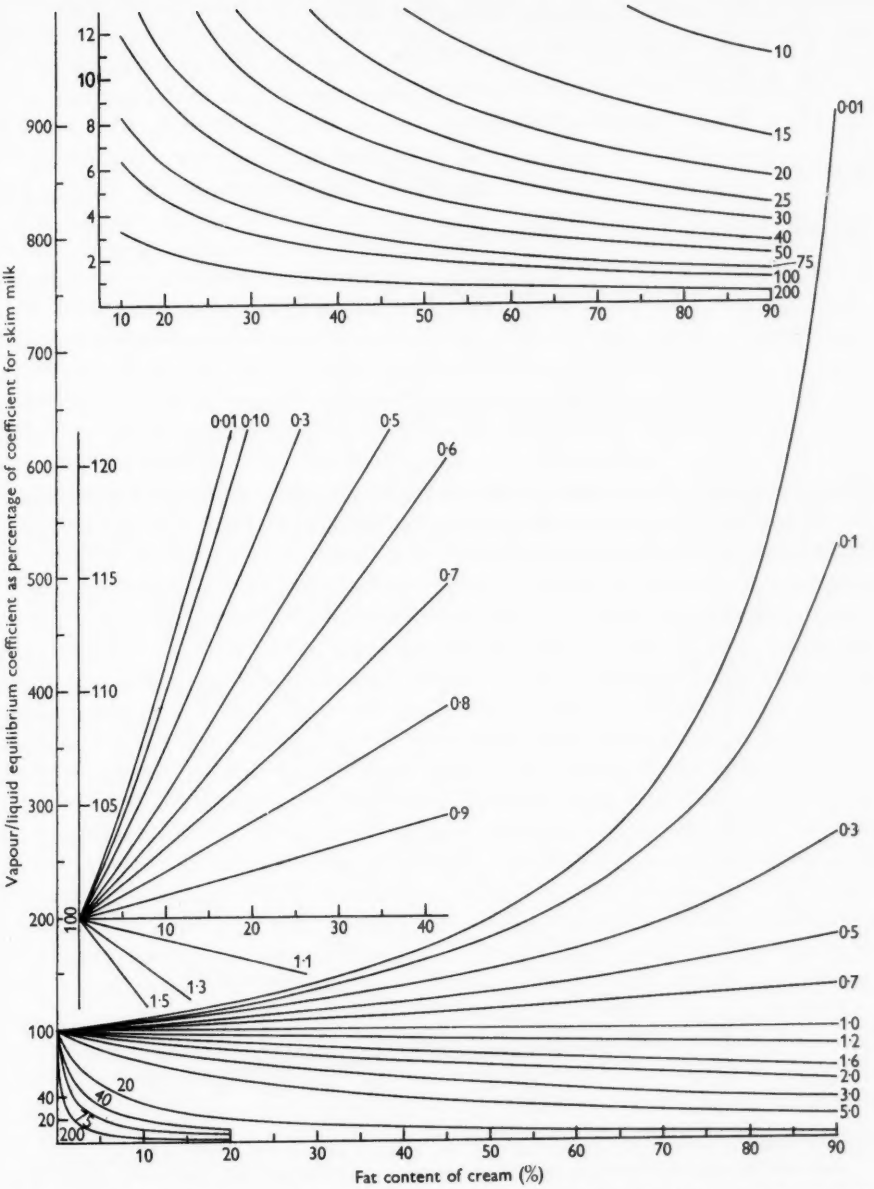

Fig. 1. Effect of fat test of cream on the vapour/liquid equilibrium coefficient for tainting substances of different butterfat/skim-milk distribution ratios. (Results expressed as percentages of the vapour/liquid equilibrium coefficient for a substance of butterfat/skim-milk distribution ratio 1:1.)

C_s = Concentration of tainting substance in the milk plasma (μg./g.).
k = Butterfat/milk-plasma distribution ratio.
f = Fat content of milk separated (%).
F = Fat content of cream obtained (%).
W = Weight of cream obtained.

Then
$$WC_c = W\frac{F}{100}C_f + W\frac{100-F}{100}C_s$$

and, using formulae I and II of Paper VII in this series[1]:

$$WC_c = W\frac{F}{100}\frac{100C_m}{f+\frac{(100-f)}{k}} + W\frac{100-F}{100}\frac{100C_m}{100-f(1-k)},$$

$$C_c = F\left\{\frac{C_m}{f+\frac{100-f}{k}} - \frac{C_m}{100-f(1-k)}\right\} + \frac{100C_m}{100-f(1-k)} \qquad \text{(II)}$$

For any one milk at a constant temperature the terms other than C_c and F in this equation are constant. Hence the relationship between fat content of the cream and content of tainting substance in the cream is linear.

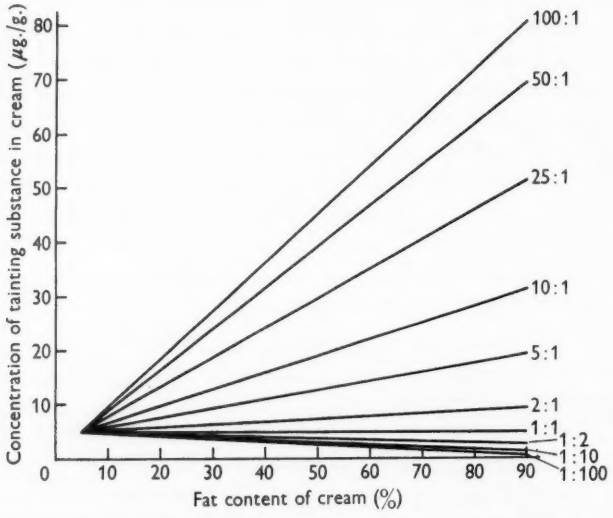

Fig. 2. Variation in concentration of tainting substance in cream separated from milk of 5% fat content and containing 5 p.p.m. of tainting substance, for different butterfat/skim-milk distribution ratios of the tainting substance at the temperature of separation.

The effect of fat content of cream separated from a milk of 5% fat content on the concentration of tainting substances in the cream is shown in Fig. 2, for a range of butterfat/skim-milk distribution ratios. When the distribution ratio is greater than 1, increase in the fat content of the cream increases the overall concentration of tainting substance in

the cream, and when the distribution ratio is less than 1 increase in fat content decreases the overall concentration in the cream. It is of interest to note that the effect of a low distribution ratio on the concentration of tainting substance in the cream is relatively smaller than the effect of a high distribution ratio, for the reason that a major proportion of the tainting substance is present in the aqueous phase of the milk and passes away in the skim-milk. A milk of 5% fat content separated to cream and skim-milk of 40 and 0·08% fat contents respectively, yields $(40–5·0)/(40–0·08) \times 100 = 87·69$ lb. of skim-milk and 12·31 lb. of cream per 100 lb.((4), p. 115). The cream contains 7·386 lb. of plasma (or skim-milk). Of the 95 lb. of plasma present in 100 lb. of original milk, therefore, 92·3% is segregated from the fat in the separation process and only 7·7% is left as cream plasma. It is clear that in milk separation substances more readily soluble in skim-milk than in butterfat are to a considerable extent eliminated in the separation process. This is in part the theoretical basis for the re-separation process for the improvement of cream and butter quality((4), pp. 157, 371). The first separation is a form of 'washing' of the cream for removal of tainting substances of low butterfat/skim-milk distribution ratio, and where substances of this type are present in milk it is of particular advantage to separate to a high-fat cream. Some of the tainting substances of high butterfat/skim-milk distribution ratio are eliminated also, but the proportion is relatively much smaller.

Effect of temperature of separation on content of tainting substance in cream

With rise in temperature some substances become relatively more soluble in butterfat than in skim-milk, i.e. the temperature coefficient of the butterfat/skim-milk distribution ratio is positive(5). When a substance of this type is present in milk, separation of the milk at a lower temperature will give a cream of lower content of tainting substance than separation at a high temperature. Conversely, when milk contains a tainting substance with a negative temperature coefficient for the distribution ratio, the separation is best done at a high temperature. Since the nature of most of the tainting substances that may be present in cream is not known, it is not possible to give to the commercial plant operator any guidance based on values for the temperature coefficients of the distribution ratios for the taints in milk. Also there may be present in the milk some substances with positive temperature coefficients of the distribution ratios and other substances with negative coefficients, or substances with the same sign for the temperature coefficient of the distribution ratios but with different magnitudes for these coefficients. Change in temperature of separation of the milk could then change the organoleptic character of the cream obtained. In the absence of basic data for the properties of tainting substances present in a cream it remains for the commercial plant operator to select the conditions of separation on the basis of a 'senses' comparative examination of creams and butters resulting from separation of the same milk at different temperatures. As will be shown later, an examination of the butter is a safer guide than an examination of the cream after treatment.

Effect of fat test of cream on content of tainting substance per unit quantity of fat

In the treatment of cream for buttermaking the cost of the deodorizing process must be related finally to the commercial product purchased, namely butterfat, or to the commercial product sold, namely butter. Thus, although the proportion of tainting substance of high butterfat/skim-milk distribution ratio in cream increases with increase in the fat

content of the cream (Fig. 2), this does not necessarily imply a greater quantity of tainting substance in the cream per unit weight of butterfat.

Concentration of tainting substances in cream of fat test F per unit weight of butterfat

$$= \frac{100C_c}{F}$$

$$= \frac{\dfrac{FC_m \times 100}{f+\dfrac{100-f}{k}} + \dfrac{(100-F)\times C_m \times 100}{100-f(1-k)}}{F} \qquad \text{(see Formula II above)}$$

$$= \frac{C_m \times 100}{f+\dfrac{100-f}{k}} + \frac{100-F}{F} \times \frac{C_m \times 100}{100-f(1-k)}. \qquad \text{(III)}$$

The first term in this equation is constant for all values of F, and represents the contribution of the tainting substance present in the fat itself, the concentration of which per unit weight of fat remains unchanged during the separation process if the temperature of the milk is not changed.

The effect of fat test of cream separated on the concentration of tainting substance in the cream per unit quantity of fat, computed by use of equation III, for different butterfat/skim-milk distribution ratios is shown in Fig. 3. For substances of low butterfat/skim-milk distribution ratios the increase of fat test of the cream results in a very marked

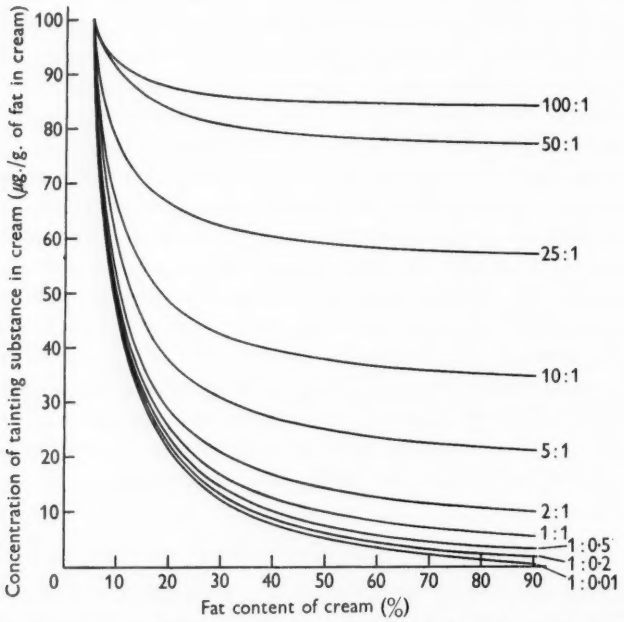

Fig. 3. Variation in concentration of tainting substance per unit weight of fat in creams of different percentage fat contents separated from milk of 5% fat content and containing 5 p.p.m. of tainting substance, for different butterfat/skim-milk distribution ratios of the tainting substance at the temperature of separation.

decrease in the content of tainting substance per unit quantity of fat. It is, of course, self-evident that elimination of some heavily taint-loaded skim-milk from the cream will markedly reduce the overall concentration of taint in the cream. But the lines in Fig. 3 for a tainting substance of high butterfat/skim-milk distribution ratio show that, despite the rise in overall concentration with rise in the fat content of cream, there is for all distribution ratios a fall in content of tainting substance in the cream per unit weight of fat in the cream.

Whatever the solubility properties of the tainting substance, therefore, it is an advantage to separate milk containing unwanted off-flavours to cream of as high a fat content as can conveniently be handled, and for substances of low butterfat/skim-milk distribution ratio the advantage of separating to an 80–90 % cream, as in the Gold'n Flow process, is very great. To make full use of the advantage of separating to a cream of high fat content, however, it is necessary to adjust the flow rates through the equipment to ensure that there is no decrease in the ratio of quantity of steam used to quantity of butterfat in the cream processed. When the equipment used for steam distillation treatment has a final vacuum-cooling section, as in the Vacreator vacuum pasteurizer, the advantage of high fat content is further increased by the extra evaporation of steam from the aqueous portion resulting from the cooling of the large proportion of butterfat present to the temperature corresponding to the vacuum applied. Advantages in taint removal are obtained both from the additional steam produced and from the concentration of the residual taint in the small volume of the aqueous portion, thus increasing the concentration in the vapour phase passing to the condenser (6).

Effect of butterfat/skim-milk distribution ratio in the churning process

The above discussion is directly applicable where all the tainting substance in the product as it leaves the steam-distillation equipment remains in the final commercial article. This holds for the deodorizing treatment of market milk and cream, the treatment of ice-cream mix, and the treatment of the butterfat/cream-plasma mixture in the Gold'n Flow continuous buttermaking process. It does not altogether hold for cream from which butter is to be made by the conventional churning process, by the Fritz continuous process, or by the Alfa continuous process, for all of which there is in the stage of conversion of cream to butter a further elimination of a portion of the aqueous phase. The extent to which a tainting substance is thus eliminated will depend on the butterfat/skim-milk distribution ratio for the substance at the churning temperature (at which a considerable proportion of the butterfat is in the solid state). The proportion of the aqueous phase of the cream retained in the butter at churning is about 10 % for washed butter (7) and 15 % for non-washed butter (8). In the manufacture of washed butter, also, there is a further possible loss by diffusion to the wash-water, the extent of which will depend on the proportion of water used, the size of the granule churned, the time of contact of wash-water with the granules, and on the butterfat/water distribution ratio for the tainting substance. These and other factors, as they relate to the application of the solubility distribution law for diacetyl and acetoin in the churning of cream to butter, have already been referred to ((4), pp. 749–53), and it was shown that the higher relative water-solubility of acetoin as compared with diacetyl results in a correspondingly greater loss to the buttermilk in the churning process. While discussion on this aspect in any detail is not relevant here, it may be noted that there is no fixed relationship between concentration of a tainting

substance in cream and concentration in the final product, butter. The proportion of the tainting substance in the cream passing into and remaining with the butter depends in the first place on the butterfat/skim-milk distribution ratio at the churning temperature, and in the second place on the fat content of the cream at conclusion of processing.

As part of a working hypothesis for use in design of equipment Scott (9) has assumed that for a tainting substance with a vapour/liquid equilibrium coefficient of 10 in 40% cream reduction of the tainting substance content of the cream to 10% of the original concentration would be a satisfactory objective (9). In the utilization of this hypothesis the possibility of variations in end-results even with substances of the average vapour/liquid coefficient postulated by Scott, owing to the effect of variation in the butterfat/skim-milk distribution ratio on the partition of the tainting substances between butter granules and buttermilk at churning, should not be overlooked. Even citation of the content of tainting substance in the cream per pound of butterfat, as illustrated in Fig. 3, would not provide a satisfactory basis for comparison of the end-result of deodorization in the cream and the end-result in the butter, especially for tainting substances relatively highly soluble in the skim-milk. The most accurate index of the likely content of tainting substance in the butter would be a citation of the content in the butterfat phase in the final cream. Assessment of the concentrations in this way, however, has little significance in any study of the cream deodorizing process itself, since during the steam distillation treatment there is a continual transfer of tainting substance from the butterfat to the aqueous phase to maintain the distribution equilibrium, and therefore for a consideration of the deodorizing process itself the concentration of tainting substance in the aqueous phase is equally as important as the concentration in the fat phase. In other words, it is necessary to compute in terms of concentration in the cream as treated.

Effect of distribution ratio on the extent of reduction in concentration of tainting substance required

It is well known that apart from the four basic taste sensations, namely, sweet, sour, bitter and salt, the differences in the flavour of foods and other products are detected by the sense of smell. It can be expected, therefore, that the vapour/liquid equilibrium coefficient of a tainting substance in the cream and butter will have a direct relationship to the intensity of the flavour sensation obtained. Because of the effect of the butterfat/water distribution ratio on the vapour/liquid equilibrium coefficient, it would be expected that the effect on flavour of cream or butter of any concentration of a substance with distribution ratio either greater or less than 1 could be very different from the effect of the same concentration of the substance on the flavour of water or 4·5% lactose solution. In the grading of butter it is the custom of many graders to smell the trier-plug before tasting it. For such an observation, apart from the basic flavour-producing property of the substance as a chemical entity, the critical property would probably be the vapour/liquid equilibrium coefficient at the temperature of observation. The effect of high butterfat/lactose-solution distribution ratio in causing a lowering of the vapour/liquid equilibrium coefficient, and therefore in increasing the difficulty of removal of a tainting substance by steam distillation treatment, could therefore at the same time lead to a lowering of the effect of the tainting substance on the flavour of the butter. This would offset to a considerable extent the lowering of the steam-volatility of the substance in the steam distillation of it from cream.

It has been shown (10) that salt in solution causes a rise in the vapour/liquid equilibrium coefficients for diacetyl and acetoin, and the magnitude of the effect is increased with increase in the salt concentration. Later work (not yet published) has established that it causes similar rises in the coefficient for the higher α-diketones. If, as seems likely, this finding is of general application, then the required extent of removal of a tainting substance by steam distillation treatment from a cream to be used for buttermaking would vary also according to the type of butter for the manufacture of which the cream is to be used. For the making of unsalted butter the cream could be less rigorously treated than for lightly salted butter, and for lightly salted butter it could be less rigorously treated than for heavily salted butter, for production of the same intensity level of flavour due to the substance in the final butter. This postulation is in line with the findings of the official dairy produce graders in New Zealand, that in the spring months, when the pastures are lush and feed taint is more prevalent in the cream, butter should not be highly salted. It is the general experience in the New Zealand butter industry, also, that unsalted butter scores somewhat higher than salted butter from the same line of cream. While some of this difference may be due to the intrinsic effect of salt on the combined flavour effect, it seems likely that it is due in part at any rate to the effect of salt in raising the vapour/liquid equilibrium coefficient of the tainting substances present in the butter. The reservation must here be made, however, that the measurements of effect of salt on the vapour/liquid equilibrium coefficient have been taken at 100° C., or, for the higher salt concentrations, at appreciably higher temperatures than 100° C. It remains to be shown experimentally that similar changes in the coefficient occur at the normal temperature of grading of butter, or at body temperature for the tasting of butter.

These aspects of the subject of butter quality control will be more fully discussed in a later paper detailing the effect of salt in solution on the vapour/liquid equilibrium coefficients of some higher α-diketones. They apply to the discussion in the present paper only to the extent of indicating that, for any one tainting substance in cream of any one percentage fat content, the concentration of tainting substance in the treated cream to be set as the desideratum, to the attainment of which the design and operation of cream processing equipment should be directed, may need to be varied according to the type of butter to be made. All that is required is to bring the concentration of tainting substance below the threshold value for detection by the grader or consumer, and it seems likely that high salt content of the butter will lower this threshold value.

SUMMARY

1. In commercial practice dairy liquids of fat contents ranging from 3 to 90% are subjected to deodorization by steam distillation treatment.

2. Increase in fat test of cream causes a rise in the vapour/liquid equilibrium coefficient for tainting substances of low butterfat/skim-milk distribution ratio, and a fall for substances of high-distribution ratio, in cream.

3. Increase in fat content of cream reduces the amount of tainting substance in the cream per unit weight of butterfat in the cream, for substances of all butterfat/skim-milk distribution ratios.

4. Separation of milk to cream of a high fat content greatly facilitates the elimination of substances of low butterfat/skim-milk distribution ratio from the cream and butter.

5. Variation in temperature of separation, by its effect on the distribution ratio for tainting substances, could affect the content of these substances in the final cream, and could possibly cause a change in the character of the cream.

6. In the partition of tainting substance between butter and buttermilk in the churning process, fat content of cream affects the proportion of residual taint passing from the treated cream into the butter.

7. It is pointed out that, because of the effect of salt in increasing the vapour/liquid equilibrium coefficient of some steam volatile substances, the threshold concentration for a tainting substance in butter can be expected to vary with variation in the rate of salting of the butter.

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FACTORS INFLUENCING THE LACTIC ACID-PRODUCING PROPERTIES OF STREPTOCOCCI USED IN THE MANUFACTURE OF CHEDDAR CHEESE

II. OBSERVATIONS RELATING SUSCEPTIBILITY WITH INSUSCEPTIBILITY

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(*Received* 28 *October* 1958)

(With 4 Figures)

In an earlier report (1), lactic acid streptococci were classified into two groups according to their growth in the presence of a heat-labile inhibitor in cow's milk. Individual strains were either 'susceptible' or 'resistant' (insusceptible) to inhibition.

Two substances are known to exist in milk which inhibit the growth of *Streptococcus pyogenes*. These have been described by Auclair & Hirsch (2) and called lactenin 1 and lactenin 2. Lactenin 2 has since been shown by Wright & Tramer (3) to be closely related to, if not identical with, the milk enzyme lactoperoxidase. Recent work, however (4), has indicated that lactenin 1 and lactenin 2 do not play any significant role in the inhibition of the lactic acid streptococci as a whole. So far, lactenin 1 appears to be specific for *Str. pyogenes*, while lactenin 2 inhibits only isolated strains of the group of lactic acid streptococci normally inhibited by raw milk. The presence of a third inhibitory substance in milk active against all 'susceptible' strains of lactic acid streptococci has now been established (4). While present in normal milk and in colostrum, it is also present in high concentration in the blood serum of the cow from which lactenin 1 and lactenin 2 are both absent.

As yet, the relationship between 'insusceptible' and 'susceptible' strains has not been clearly defined. Two strains of *Str. cremoris*, lysed by the same phage race, were reported to differ in respect to their growth in raw milk (1). More recently, Czulak & Naylor (5) observed that, in contrast to the parent strain (a strain of *Str. lactis*), which was insusceptible to inhibition, the secondary growth appearing after phage lysis was inhibited by raw milk.

The present investigation, which was concerned with the effect continuous growth in autoclaved milk might have on 'insusceptible' strains of lactic acid streptococci, provides evidence that the 'susceptible' strain is a variant of the 'insusceptible' type.

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EXPERIMENTAL

Milk used in these studies was drawn from the bulked milk of a herd of mixed breeds with individual cows in varying stages of lactation. Where used, pasteurization was carried out by the H.T.S.T. method in which milk was heated to a temperature of 72·7°C for 15 sec. To destroy the inhibitory properties of milk, samples were heated to 100° C. for 20 sec. (boiled).

A brief history of the organisms used in this investigation, strains of *Str. lactis* and *Str. cremoris*, has been given elsewhere(1). All strains were maintained in continuous growth by daily subculture in 10 ml. quantities of autoclaved skim-milk (12 lb./in.^{2} for 20 min.) and incubated at 25° C. for 24 hr. Each week, individual strains were inoculated into raw (unheated), pasteurized, and boiled milk, using a 9% inoculum. After incubation at 30° C. for 6 hr., the extent of acid production, which was taken as a measure of growth, was determined directly by titration with 0·1 N-NaOH and expressed as percentage lactic acid. Mean values for the lactic acid produced by each strain were determined for each four-weekly period. These values are shown in the accompanying figures.

The 'insusceptible' strain

A strain insusceptible to inhibition shows little variation in acid production whether grown in the presence or absence of the inhibitory substances of milk. As shown in Fig. 1, the rate of growth of *Str. cremoris*, Strain C3, is approximately the same in raw, pasteurized, and boiled milk. The organism remained insusceptible to inhibition throughout the entire test period.

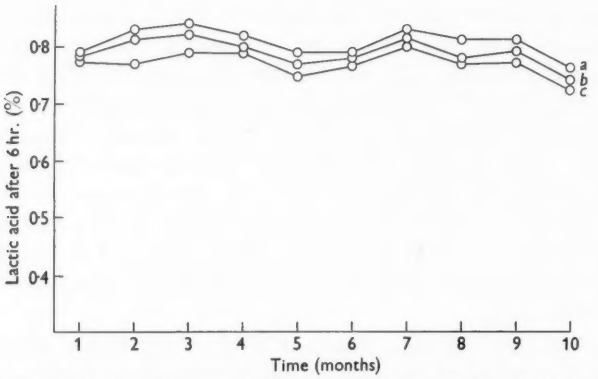

Fig. 1. Lactic acid production by an 'insusceptible' organism, *Str. cremoris*, Strain C3, maintained by daily subculture in autoclaved milk and tested weekly in *a* boiled, *b* pasteurized, and *c* raw milk. Data given represent the mean values obtained for each 4-weekly period.

Transition from insusceptibility to susceptibility

From the weekly data, it became apparent that many strains originally insusceptible to inhibition by raw milk were gradually losing their capacity to grow in this medium. As shown in Fig. 2, the rate of growth of *Str. lactis*, Strain C10, became progressively slower in raw and pasteurized milk, but remained unchanged in boiled milk. Periodic testing against a phage race specific for this strain showed that no change in the phage

relationship of the organism had occurred. As a check against continuous growth in autoclaved milk, all strains were refrigerated at 0·5° C. in chalk litmus milk and sub-cultured only once every 3 months. Samples were withdrawn at weekly intervals from the individual cultures and tested in the manner already described. Under these conditions 'insusceptible' strains (for example, *Str. lactis*, C10) showed no obvious change in their rate of acid production in raw and pasteurized milk.

The rate at which an organism becomes susceptible to inhibition by raw milk, as a result of continuous growth in autoclaved milk, was observed to differ for each individual strain. Some strains became fully susceptible within a few weeks, others took several months.

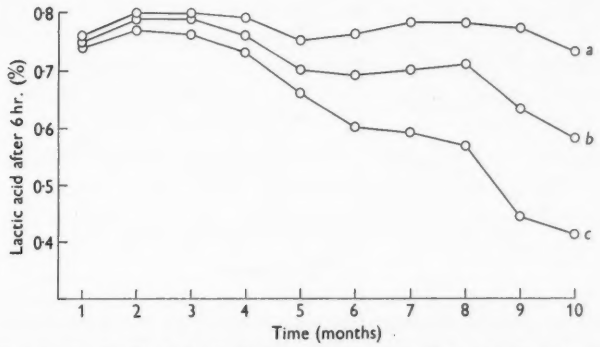

Fig. 2. Lactic acid production by *Str. lactis*, strain C10, maintained by daily subculture in autoclaved milk and tested weekly in *a* boiled, *b* pasteurized, and *c* raw milk. Data given represent the mean values obtained for each 4-weekly period. The strain, originally insusceptible, has become suceptible to inhibition by raw and pasteurized milk.

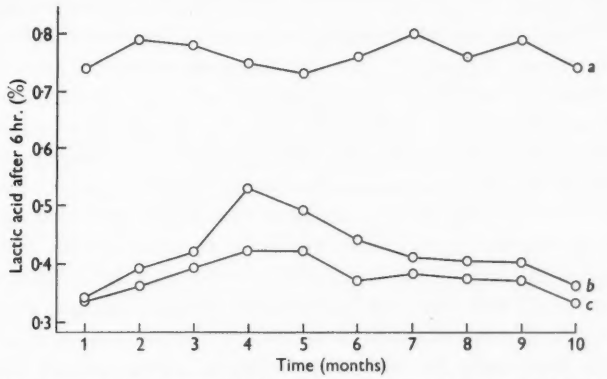

Fig. 3. Lactic acid production by a 'susceptible' organism, *Str. cremoris*, strain ML1, when maintained by daily subculture in autoclaved milk and tested weekly in *a* boiled, *b* pasteurized, and *c* raw milk. Data given represent the mean values obtained for each 4-weekly period.

The 'susceptible' strain

A strain which has become fully 'susceptible' shows marked inhibition when grown in raw and pasteurized milk, but not in boiled milk where the rate of growth appears to equal that of the original 'insusceptible' strain. The response obtained with *Str. cremoris*, Strain ML1, as shown in Fig. 3, is characteristic of all strains which have become fully

susceptible to inhibition. Once susceptible to inhibition, an organism appears to remain in that condition indefinitely.

Earlier observations (1) suggested that 'susceptible' strains differed in the degree to which they were inhibited by raw milk. This difference is well illustrated in Fig. 4 where all three strains examined grew equally well in boiled milk but differed, quite markedly, in their rate of growth in raw milk.

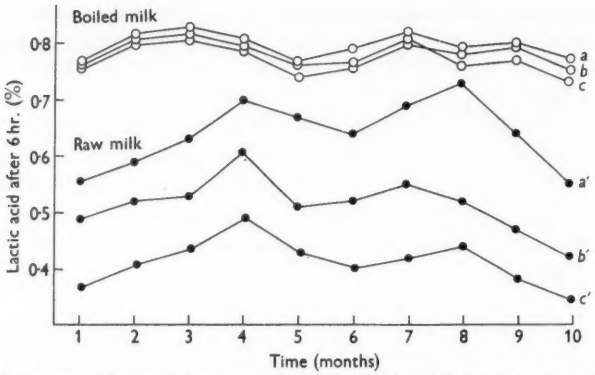

Fig. 4. Variation in the suceptibility of three strains of *Str. cremoris* to inhibition by raw milk. The organisms were maintained in autoclaved milk and tested weekly in boiled, pasteurized and raw milk. Data given, for boiled and raw milk, represent the mean values obtained for each 4-weekly period. Strain R6 (*a*–*a*′); strain ML4 (*b*–*b*′); strain HP (*c*–*c*′).

DISCUSSION

When lactic acid streptococci, in the wild state, are first isolated from raw milk by plating-out procedures, the majority of organisms isolated are insusceptible to inhibition by raw milk, i.e. their rate of growth in raw and in autoclaved milk is essentially the same under the experimental conditions used. However, in the case of many strains, continuous growth in autoclaved milk leads to the recovery by plating of an ever-increasing proportion of organisms, the growth of which is markedly inhibited in raw milk. This evidence, supported by the observations of other investigators (see, for example, Czulak & Meanwell (6), Garvie & Mabbitt (7)), suggests that lactic acid streptococci produce variants which are inhibited in raw milk, but not in autoclaved milk. If the production of such variants is a constant characteristic of these organisms, then these variants would also arise in the wild state but would never become dominant since they are inhibited by raw milk. The rate at which these variants are produced could account for the variation in the time taken by individual strains to change to the fully susceptible condition.

It is well recognized throughout the cheese industry that starter organisms, maintained in the laboratory, often lose their capacity to produce acid rapidly in H.T.S.T. pasteurized milk. Jensen (8) observed that 'some pure strains showed a tendency over a considerable period of time to suffer a gradual loss in activity while other strains proved remarkably stable and could be relied upon to behave true to type almost indefinitely'. In an endeavour to prevent this loss of activity by starters, many laboratories adopt one of the following two, opposing, procedures. (1) Individual strains are maintained in con-

tinuous growth in autoclaved milk by daily subculture, with periodical plating-out and re-selection of cells having the same morphological characteristics and activity (in autoclaved milk) as the parent strain. (2) Individual strains are kept dormant by refrigeration or freeze-drying. Strains are kept in readiness for immediate use by daily subculture in autoclaved milk. A limit is placed on the number of daily transfers, after which the culture is discarded and restarted afresh from the dormant stock. The results of the present investigation clearly support the latter procedure as a means of retarding the changes occurring in autoclaved milk which render strains susceptible to inhibition. Selection of strains slow to change to the 'susceptible' condition when grown in autoclaved milk would be an additional safeguard. Continuous growth in autoclaved milk as described in (1) above could only be justified if, after plating-out, strains were selected on the basis of their high activity in both raw and autoclaved milk.

As H.T.S.T. pasteurization does not destroy the inhibitory properties of milk, loss of susceptibility to inhibition inevitably leads to the rejection of particular starters because of their inability to produce acid rapidly.

The maxima shown by the curves in Fig. 4 would seem to be related to the milk supply as influenced by seasonal conditions. It has frequently been observed that inhibition reaches a maximum during periods when pastures are in a state of decline.

SUMMARY

1. Continuous growth of lactic acid streptococci in autoclaved milk allows strains, originally insusceptible, to become susceptible to inhibition by raw milk.

2. Variants of the original organisms, the growth of which is inhibited in raw milk but not in autoclaved milk, appear to predominate in strains maintained in autoclaved milk.

3. The time required to allow these changes to occur in autoclaved milk varies with the strains used.

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CITRIC ACID CONTENT OF MILK IN MANITOBA*

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(With 1 Figure)

Though important, citric acid seems to be one of the least investigated of the minor constituents of milk. Henkel (1) isolated and identified citric acid as a normal constituent of cow's milk as early as 1888. A search of the literature reveals, however, that at the present time, 70 years later, there are still too few published results of investigations to get a true picture of the citric acid content of milk. The available data are summarized in Table 1.

Table 1. *Published values for citric acid content of milk*

Authority	No. of samples	and description	Average citric acid content (%)	Highest value (%)	Lowest value (%)
Scheibe (2)	32	—	0·132	0·298	0·082
Sommer & Hart (3)	16	—	0·231	0·248	0·191
Hess, Unger & Supplee (4)	2	Cows stall fed	0·08	—	—
		Cows pasture fed	0·130	—	—
Supplee & Bellis (5)	17	Winter and summer	0·145	0·182	0·121
		Winter	0·142	—	—
		Summer	0·148	—	—
	8	Evaporated and condensed milks, reconstituted	0·112	0·151	0·078
	15	Milk powder, reconstituted	0·137	0·177	0·102
Kieferle, Schwaibold & Hackmann (6)	104	Individual quarter samples from 26 cows	—	0·4	0·2
Sherwood & Hammer (7)	335	From individual cows in various stages of lactation, of various breeds and on various feeds	0·18	0·33	0·07
Hartman & Hilling (8)	58	Bulk supplies of 14 cities	0·160	0·190	0·140
Rumments (9)		Cows, mid-lactation	0·213	—	—
		Same cows towards end of lactation	0·134	0·180	0·038
Arup (10)	25	—	—	0·206	0·150
	11	Defatted milk solids, reconstituted	—	0·184	0·151
Heinemann (11)	27	Defatted milk solids, dry wt. basis	1·995	2·09	1·88
Babad & Shtrikman (12)	8	—	0·193	0·245	0·180
	2	Milk powder, dry wt. basis	2·52	—	—
			1·91	—	—

The present investigation was a part of a study of the composition of milk in Manitoba. The purpose was to obtain information on the level of citric acid in milk in Manitoba and its variability. The study was carried out on bulk milk over the period January to December 1955, and on the mixed milk of the University Holstein herd and on the milk of individual cows from this herd over the period April 1956 to March 1957.

* A study supported by the Canada Department of Agriculture.

EXPERIMENTAL

The samples of bulk milk were obtained from the three largest dairies in the City of Winnipeg. The samples of mixed milk of the University herd were taken at fortnightly intervals. In addition, samples were taken on 5 days each week during 8 weeks in May and June and during 10 weeks in September to November at the time the feeding of the cows in the herd was changed from stall feeding to pasture and vice versa. Samples were also taken at weekly intervals throughout the lactations of three individual cows in the University herd. In a further experiment samples were taken once a week from the milk of each of five cows, for 12 weeks at the beginning and 12 weeks at the end of lactation.

For the determination of the citric acid content of milk a modification of the colorimetric method of Saffran & Denstedt was used. A description and discussion of the method is reported elsewhere (13).

Table 2. *Citric acid content of bulk milk and of milk of the University Holstein herd*, %

	Dairies 1955			Univ. of Man. herd milk
	1	2	3	1956–57
Jan. I	0·185	0·181	0·176	0·182
II	0·174	0·174	0·175	0·176
Feb. I	0·175	0·178	0·172	0·185
II	0·169	0·172	0·174	0·186
Mar. I	0·173	0·176	0·173	0·175
II	0·171	0·162	0·162	0·175
Apr. I	0·169	0·176	0·174	0·165
II	0·177	0·180	0·178	0·153
May I	0·175	0·182	0·186	0·164
II	0·182	0·181	0·174	0·157
June I	0·171	0·164	0·159	0·151
II	0·179	0·176	0·172	0·158
July I	0·165	0·166	0·161	0·159
II	0·164	0·166	0·164	0·184
Aug. I	0·162	0·165	0·160	0·142
II	0·165	0·164	0·162	0·155
Sept. I	0·167	0·171	0·175	0·160
II	0·175	0·176	0·178	0·184
Oct. I	0·175	0·176	0·175	0·159
II	0·168	0·173	0·173	0·169
Nov. I	0·180	0·174	0·173	0·153
II	0·170	0·168	0·171	0·181
Dec. I	0·162	0·176	0·177	0·173
II	0·162	0·169	0·172	0·176
Average	0·171	0·173	0·172	0·168
Range	0·159–0·186			0·142–0·186
S.D.		0·006		0·013
C.V.		3·49		7·74

RESULTS AND DISCUSSION

The numbers of samples analysed were, bulk milk, 72; mixed herd milk, 114; individual cow's milk, 254.

The average citric acid content of the bulk milk (Table 2) was 0·172% with a minimum of 0·159% and a maximum of 0·186%. The average citric acid content of the herd milk was 0·168% with a minimum of 0·142% and a maximum of 0·186%. The analysis

Table 3. *Analysis of variance*

Source	D.F.	M.S.
Dairies and Univ. herd	3	0·000121
Housing and grazing seasons	1	0·001023*
Error	91	0·000062
Total	95	—

* $P < 0{\cdot}01$.

Table 4. *Citric acid percentage in the milk of individual cows during a lactation period*

Cow ...	Cl	W	T
No. of samples ...	53	53	50
Colostrum*	0·093	0·110	0·118
Average (colostrum incl.)	0·171	0·179	0·189
Range in normal milk	0·136–0·219	0·135–0·233	0·159–0·231
Standard deviation	0·023	0·024	0·019
Coefficient of variation	13·5	13·4	10·1

Differences between average values

Cl–W	0·008±0·005	(104 D.F.)	$P > 0{\cdot}05$
Cl–T	0·018±0·004	(101 D.F.)	$P < 0{\cdot}01$
W–T	0·010±0·004	(101 D.F.)	$0{\cdot}05 > P > 0{\cdot}01$

* Sample taken from second and third milking after calving.

Table 5. *Coefficients of variation of various constituents of milk in Manitoba*

	Bulk milk	Herd milk	Milk of indiv. cows
Fat	3·97	5·33	16·02*
Protein	4·05	3·71	14·95*
Lactose	1·57	3·63	5·61*
Ash	1·51	2·60	6·65*
Calcium (CaO)	2·00	5·75	—
Total phosphorus (P_2O_5)	3·40	4·55	—
Magnesium (MgO)	8·33	10·00	—
Chlorine (Cl)	1·85	9·65	—
Citric acid	3·49	7·74	10·6–13·5

* Reported by Overman *et al.* (14).

of variance (Table 3) indicates that the differences between the citric acid contents of the bulk milk from the different dairies and that of the milk of the University herd are not significant ($P > 0{\cdot}05$). The average citric acid content of the milk of 3 individual cows (Table 4) for whole lactations were: 0·171, 0·179 and 0·189%. The difference between the citric acid contents of the milks of cows Cl and W is not significant ($P > 0{\cdot}05$), but the difference for the milks of cows W and T is significant ($P < 0{\cdot}05$), and the difference for the milks of cows Cl and T is highly significant ($P < 0{\cdot}01$).

The average citric acid content of all these milks was quite uniform. However, the variations in their citric acid contents were different. The coefficient of variation of herd milk (7·74) was over twice the coefficient of variation in bulk milk (3·49). The coefficients of variation of the milk of individual cows (13·5, 13·4 and 10·6) were approximately three times the coefficient of variation of the bulk milk.

Table 5 gives the coefficients of variation of various constituents of milk as found in this laboratory. It may be seen that in bulk milk the variations in citric acid content are similar to the variations in phosphorus content but smaller than the variations in fat, protein and magnesium contents and larger than the variations in the contents of the other constituents. In mixed herd milk the variations in the citric acid content were

smaller than the variations in magnesium and chlorine contents, but larger than those in the contents of other constituents. For the milk of individual cows, results on the citric acid content are lacking. However, compared with the results reported by Overman, Sanman & Wright (14) for the major constituents of the Holstein milk, the variations in the citric acid content were smaller than the variations in fat and protein contents but larger than those in lactose and ash contents.

The citric acid content of milk varies from month to month (Table 2). In the present study the variations also showed a seasonal character. The citric acid content of milk in the grazing season was slightly lower and somewhat more variable than in the housing season. The differences were small, averaging 0·005% in bulk and 0·012% in herd milk,

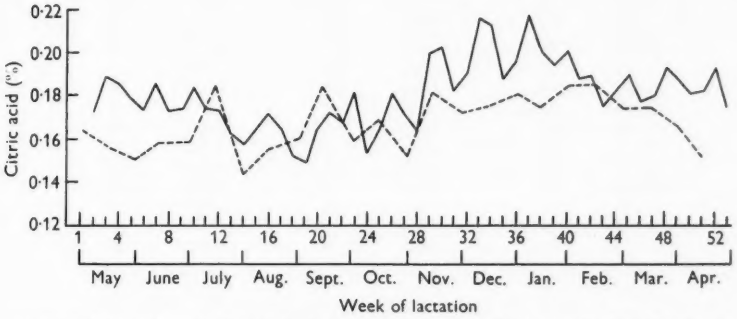

Fig. 1. Average citric acid contents of mixed herd milk (----) and mixed milk from 3 cows (———) during 1 year.

but significant ($P < 0·01$) (Table 3). This is in disagreement with some earlier reports (4) which stated that summer feeding increases the citric acid content of milk. To study more closely the effect of change of feeding of cows from stall feeding to pasture and reverse, samples of herd milk were examined five times weekly in spring and autumn before and after changes in feeding of the cows. Altogether 40 samples were examined in the spring and 50 in the autumn. In all these 90 spring and autumn milk samples the citric acid content ranged from 0·142 to 0·193% and averaged 0·167%. The standard deviation was 0·012 and the coefficient of variation 7·38. These values are almost identical to those given for the herd milk in Table 2. Table 6 gives the citric acid content of the milk of 14 samples before and 14 samples after the change of feeding of the cows in spring and autumn. It can be seen that in spring, after the cows went to pasture, the citric acid content of milk decreased and in autumn, after they returned to stall feeding, it increased. A 't' test reveals that the decrease in spring was significant ($0·05 > P > 0·01$) and the increase in autumn was not ($P > 0·05$).

Fig. 1 shows the variations in the average citric acid content of weekly samples of milk from 3 individual cows throughout the lactation period together with the citric acid content of the mixed herd milk in the same period. It may be seen that the citric acid content of milk was similar at the beginning and at the end of lactation. It was lower in the summer and early autumn months and higher in winter. Table 7 shows the citric acid content of the milk of 5 individual cows during 12 weeks at the beginning and 12 weeks at the end of lactation period. It can be seen that there are some fluctuations in the citric acid content of milk in the different periods; however, these fluctuations do not show any

regularity or relationship with the stage of lactation. Further, it can be seen that the variations in the citric acid content of milk during the lactation are different in the milk of different cows. The citric acid content in the milk of the cows Ap and Sh was remarkably lower at the end of lactation than at the beginning. The reason may be that the lactation periods of these cows ended in summer when the citric acid content of the milk was

Table 6. *Citric acid content of milk of the University Holstein herd, stall and pasture feeding*

Stall feeding		Pasture		Pasture		Stall feeding	
Date	%	Date	%	Date	%	Date	%
10 May	0·167	5 June	0·170	23 Oct.	0·175	10 Nov.	0·171
11	0·158	6	0·155	24	0·187	12	0·192
12	0·169	7	0·151	25	0·189	13	0·186
15	0·157	8	0·155	26	0·187	14	0·181
16	0·164	12	0·166	27	0·173	15	0·175
17	0·170	13	0·164	30	0·187	16	0·176
18	0·168	14	0·174	31	0·171	19	0·175
19	0·175	15	0·165	1 Nov.	0·153	20	0·166
22	0·174	19	0·158	2	0·153	21	0·178
23	0·159	20	0·158	3	0·156	22	0·172
24	0·154	21	0·155	5	0·149	23	0·172
25	0·166	22	0·147	6	0·163	24	0·193
26	0·163	26	0·152	7	0·157	26	0·173
29	0·177	27	0·147	8	0·152	27	0·173
Average	0·166		0·158		0·168		0·177
S.D.	0·007		0·008		0·015		0·008

Difference between average values

Stall feeding – pasture
$0{\cdot}008 \pm 0{\cdot}003$ (26 D.F.) $0{\cdot}05 > P > 0{\cdot}01$

Pasture – stall feeding
$-0{\cdot}009 \pm 0{\cdot}005$ (26 D.F.) $P > 0{\cdot}05$

Table 7. *Citric acid content of milk of individual cows at the beginning and end of lactation*

Cow ...	Cl		W		T		Ap		Sh	
	Month	%	Month	%	Month	%	Month	%	Month	%
Beginning of lactation	May	0·177	June	0·176	June	0·192	Jan.	0·195	Jan.	0·203
	June	0·169	July	0·170	July	0·192	Feb.	0·197	Feb.	0·186
	July	0·160	Aug.	0·174	Aug.	0·188	Mar.	0·201	Mar.	0·168
End of lactation	Feb.	0·164	Mar.	0·183	Feb.	0·204	July	0·168	July	0·157
	Mar.	0·167	Apr.	0·200	Mar.	0·200	Aug.	0·147	Aug.	0·159
	Apr.	0·195	May	0·169	Apr.	0·192	Sept.	0·158	Sept.	0·160

Each figure is an average of 4 samples in a 4-week period.

lowest. The variations in the citric acid content of the milk of individual cows in general are similar to the variations in the citric acid content of the mixed herd milk. The citric acid content of the colostrum was remarkably lower than in normal milk. In the colostrum of these 5 individual cows it varied from 0·093 to 0·118%.

The citric acid content of the evening and morning milk of 5 individual cows was examined in five 2-day periods (Table 8). The average content was, in 50 samples of evening milk, 0·166% and in 50 samples of morning milk 0·167%. The standard deviations were 0·021 and 0·020 respectively. Thus the citric acid content was similar in both milks. This indicates that the time of the milking had no influence on the citric acid content of milk. In the citric acid content of evening and morning milk from two successive milkings of one individual cow, however, there were remarkable differences. The highest difference in this experiment was 0·034% or 23·6% of the lowest value.

Table 8. *Citric acid content of evening and morning milk of individual cows*

Cow ...	Cl		T		W		Ap		Sh	
Date of sampling	E.	M.	E.	M.	E.	M.	E.	M.	E.	M.
26–27 June	0·141	0·151	0·184	0·187	0·156	0·160	0·132	0·136	0·144	0·145
27–28	0·145	0·142	0·193	0·181	0·157	0·152	0·136	0·142	0·145	0·147
3–4 July	0·158	0·168	0·205	0·195	0·176	0·174	0·172	0·162	0·164	0·183
4–5	0·157	0·158	0·178	0·181	0·178	0·177	0·144	0·157	0·169	0·169
18–19 July	0·149	0·149	0·210	0·200	0·188	0·173	0·160	0·169	0·155	0·182
19–20	0·151	0·150	0·199	0·170	0·188	0·156	0·143	0·152	0·195	0·189
20–21 Aug.	0·171	0·167	0·199	0·189	0·189	0·160	0·182	0·198	0·186	0·164
21–22	0·145	0·153	0·168	0·190	0·145	0·138	0·156	0·158	0·148	0·139
4–5 Sept.	0·155	0·153	0·194	0·217	0·143	0·172	0·144	0·178	0·144	0·160
5–6	0·153	0·170	0·212	0·218	0·157	0·159	0·180	0·175	0·154	0·139

Average: Morning milk = 0·167%
Evening milk = 0·166%

S.D.: Morning milk = 0·020
Evening milk = 0·021

C.V.: Morning milk = 12·00
Evening milk = 12·65

Difference between average values
Morning – evening milk 0·001 ± 0·004 (98 D.F.) $P > 0·05$

SUMMARY

The citric acid content of 440 samples of milk has been determined. The average citric acid content of bulk milk of an estimated 10,000 cows was 0·172%. The average citric acid content of the mixed milk of a Holstein herd of 35 cows was 0·168%, and the average citric acid content of the milk of individual cows was 0·171–0·189%. The coefficients of variation of the citric acid content were: in bulk milk 3·49, in mixed herd milk 7·74 and in the milk of individual cows 10·6–13·5. The citric acid content of the milk of individual cows may differ significantly. The citric acid content of milk in the grazing season was slightly lower and more variable than in the housing season. The stage of lactation had no significant effect on the citric acid content of milk. The time of day of the milking had no influence on the citric acid content of milk. Marked differences may occur in the citric acid content of milk from two successive milkings of an individual cow, and in this study differences up to 0·034% or 23·6% of the lowest value have been found.

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THE COMPOSITION OF SOW'S MILK DURING LACTATION WITH PARTICULAR REFERENCE TO THE RELATIONSHIP BETWEEN PROTEIN AND LACTOSE

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(*Received* 21 *November* 1958)

(With 2 Figures)

In addition to the changes in composition which occur during the transition from colostrum to normal milk (1), the percentages of the major constituents of sow's milk change during the course of lactation. Few workers have sampled milk from the same sows frequently and regularly throughout lactation, but those who have done so (2, 3, 4, 5, 6, 7, 8, 9) generally agree that ash percentage rises steadily, protein percentage tends to decline until the second or third week and then rises, while the percentage of lactose tends to decline steadily throughout lactation. The fat percentage is evidently very variable and fluctuates widely from week to week and possibly from day to day. Some workers have reported a tendency for fat percentage to decline (2, 3, 4, 5, 7, 8, 10), and some a tendency for it to rise (6, 11, 12, 13), while others (14) could observe no significant trend either way.

In an experiment at the Rowett Institute, to determine the influence of level of total food intake upon milk production in sows, data have been obtained on the composition of milk samples obtained regularly during the course of twenty-four lactations. These are here presented as a contribution to the meagre knowledge of the variations which occur in the composition of sow's milk with stage of lactation and between sows. The influence of level of feeding upon milk yield and composition have been reported elsewhere (15).

EXPERIMENTAL

Milk samples were obtained from each of eight Wessex Saddleback sows for the first, second and third 8-week lactations. Samples were taken by milking machine after Pitocin injection (16) on the 4th day after parturition and at weekly intervals thereafter. Before milking the piglets were removed from the sow for $1\frac{1}{4}$–$1\frac{1}{2}$ hr., so that the amount obtained by machine from all functional teats was similar to that obtained by the piglets at one suckling, i.e. from 300 to 500 ml.

After being filtered through linen to extract any foreign particles each milk sample was stored at 1° C. for not more than 1 week before analysis. Protein was determined by the macro-Kjeldahl method (N × 6·38), fat by the Gerber method, lactose by McDowell's adaptation (17) of Lane & Eynon's method and dry matter and ash by the methods described by Ling (18).

The milking procedure did not appear to affect adversely litter growth rates; the mean individual weaning weight at 8 weeks was 38·5 lb., and all sows reared either 8, 9 or 10 piglets per litter.

RESULTS

General composition

Mean values for the 24 lactations are presented in Fig. 1. The percentages obtained during the first week, i.e. the 4th day, of lactation suggest that the transition from colostrum to normal milk was not complete by that time. After the transition, which must have finished some time between the 4th and 10th days, the percentages of ash and protein rose

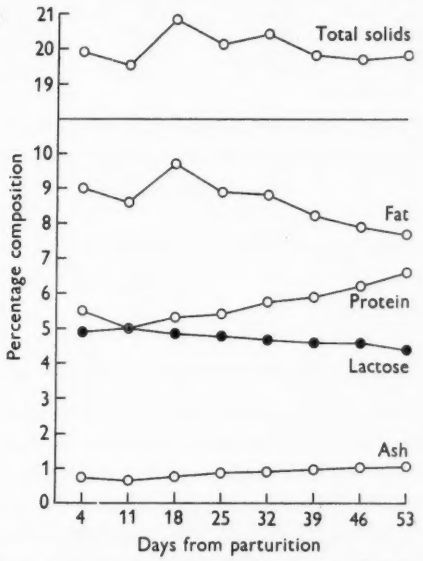

Fig. 1. Percentage composition of sow's milk, means of 24 lactations.

steadily and the percentage of lactose fell. Throughout lactation fat was the major constituent of the solids and closely influenced the percentage of dry matter. Although the milk of individual sows showed considerable variation in fat content from week to week, e.g. individual samples were as high as 13·9% and as low as 4·9%, there was, as shown by the mean curve, a tendency for the fat percentage to reach a peak in the 3rd week and then decline with advance in lactation.

Differences between sows

The mean compositions of 24 milk samples (8 weekly samples from each of 3 lactations) from each sow are shown in Table 1. As in any one sow during lactation so between sows over the whole lactation, of the major constituents of the milk, fat percentage showed the greatest variation.

Differences between successive lactations

Mean percentage compositions of the milk of all sows for each lactation are shown in Table 2. There were no marked differences between lactations but there was an indication that milk of the first lactation may differ slightly in the percentages of the major constituents from that produced subsequently.

Table 1. *The percentage composition of milk from* 8 *sows; means of* 24 *samples from each sow, taken on eight occasions at weekly intervals during each of three lactations*

	Milk constituent				
Sow	Total solids	Protein (N × 6·38)	Lactose	Fat	Ash
2	18·6	5·3	4·9	7·4	0·87
8	20·0	5·4	4·9	8·7	0·85
9	19·0	5·5	5·0	7·7	0·84
16	20·2	5·7	4·8	8·7	0·87
5	20·3	5·9	4·7	8·8	0·90
6	21·5	6·1	4·3	10·0	0·97
10	20·5	6·0	4·5	8·9	0·89
15	20·1	5·9	4·8	8·4	0·92

Table 2. *The average percentage composition of the milk from* 8 *sows at each of three successive lactations; means of* 64 *samples,* 8 *from each sow taken at weekly intervals*

	Milk constituent				
Lactation	Total solids	Protein (N × 6·38)	Lactose	Fat	Ash
1st	19·8	5·5	4·9	8·5	0·88
2nd	20·2	5·8	4·6	8·9	0·90
3rd	20·0	5·8	4·7	8·5	0·90

Relationship between protein and lactose

Two examples of the protein and lactose curves of individual sows in each lactation are shown in Fig. 2. Percentage protein and percentage lactose in the milk of any particular sow in any particular week showed considerable fluctuation from the mean values for that sow, for that week or that lactation, but when one was relatively high the other almost always tended to be relatively low. The relationship between these fluctuations for all 24 lactations was significantly different ($P < 0{\cdot}01$) from the relationship between changes in percentages of protein and lactose with stage of lactation. After elimination of this time effect there was still a highly significant negative correlation ($r = -0{\cdot}62$; $P < 0{\cdot}001$) between the percentages of protein and lactose in the sows' milk.

DISCUSSION

For purposes of comparison with the mean values obtained in the present work, mean percentage compositions of sow's milk determined by some other workers are presented in Table 4. Data obtained on composition trends with advance in lactation agree generally with the findings of previous workers, and the inverse relationship between percentages of protein and lactose with advance in lactation (Fig. 1) has been reported previously in relation to cows (19, 20). The writer has been unable, however, to find any previous reference to the inverse relationship found to exist between the percentages of protein and lactose in sow's milk independently of normal lactation trends.

Data published by Tocher in 1925 (19) showed a clear inverse relationship in cow's milk between the percentages of lactose and albumen nitrogen but not between lactose and casein nitrogen. As non-casein nitrogen forms a much larger part of the total nitrogen of sow's milk than it does in the milk of the cow (21), any relationship between lactose and non-casein nitrogen may be expected to be more obvious in sow's milk.

This relationship between the percentages of protein and lactose in sow's milk has been commented upon in a previous paper(22), but data are now available for a greater number of sows and lactations. The reason for it is not clear, but two possible explanations may be postulated: (1) both milk protein and lactose may be formed from amino acids

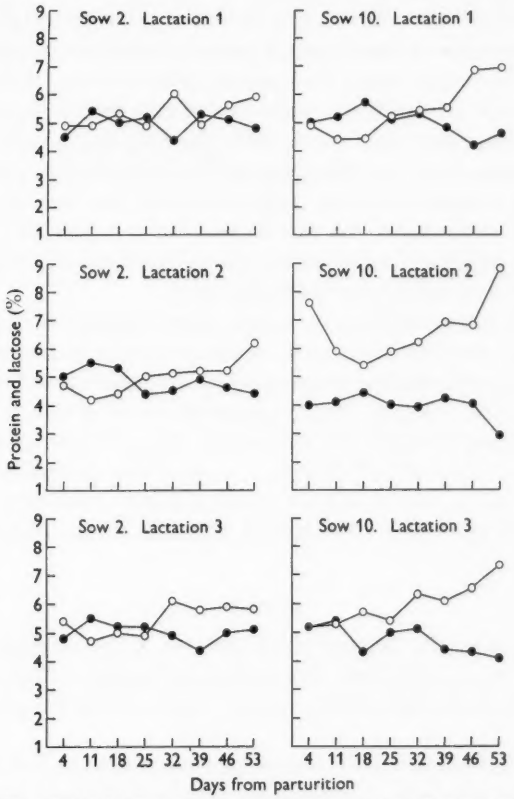

Fig. 2. The relationship between percentages of protein and lactose in the milk of sows 2 and 10 over three lactations. o——o Protein; •——• lactose.

Table 3. *Mean percentage composition of sow's milk according to authorities who sampled frequently and regularly throughout lactation*

Authority	Breed	No. of sows	No. of samples	Percentage composition: Total solids	Protein	Lactose	Fat	Ash
Braude *et al.*(2)	Large white	9	40–76	19·87	5·79	4·81	8·17	0·94
Bowland *et al.*(3)	Poland China, Chester White	—	134	20·08	7·26	5·13	6·81	0·99
Heidebrecht *et. al.*(5)	Hampshire, Duroc Jersey, Chester White	35	22	19·7	6·0	—	8·0	1·03
Niwa *et al.*(6)	Middle White	4	44	20·32	5·26	4·18	9·97	0·91
Smith(7)	Berkshire	5	40	—	6·2	5·0	7·7	—
Berge & Indrebø(14)	Norwegian-Landrace	11	16	17·5	5·4	5·9	6·1	0·8
Perrin(9)	Berkshire	44	450	21·23	6·11	4·62	9·58	0·92
Present work	Wessex-Saddleback	8	192	20·0	5·7	4·7	8·6	0·89

from the diet and, possibly, also from body tissues—protein directly and lactose from glucogenic residues after deamination; (2) both the precursors of milk protein, i.e. amino acids, and of lactose, i.e. blood glucose and glucose freed from gluco-proteins in the synthesis of milk proteins, may supply energy to the mammary gland for milk secretion and one may exert a sparing effect on the other.

For all the sows in the present work the amount of crude protein fed during lactation should have been adequate or more than adequate to meet protein requirements, and yet nearly every sow lost weight during that period. It would seem, therefore, that dietary energy was insufficient to meet the requirements of milk production and it may be considered safe to assume that amino-acids from the diet, and possibly also from body tissues, were deaminated and the glucogenic residues converted to carbohydrate for the provision of energy in addition to that yielded from body fat. The fact that milk protein and lactose were inversely related but that fluctuations in lactose content were less great, suggests that the synthesis of some lactose was independent of protein synthesis. This may have been lactose formed from blood glucose.

To summarize, it is suggested that in the sow, where weight loss during lactation is the rule rather than the exception, deaminated amino-acids may be precursors of milk sugar —either directly, or indirectly by supplying energy otherwise provided by blood glucose. This hypothesis does not conform with the generally accepted view that blood glucose is the principal precursor of lactose[23] but receives support from evidence obtained by various workers, and reported by Folley[24], to suggest that the mammary gland of some non-ruminant species, i.e. the rat and the mouse, is able to deaminate amino acids taken up by the blood and that this is an important process in the metabolism of the lactating gland.

SUMMARY

1. Data are given on the changes which occurred in the composition of sow's milk during the course of 24 lactations. The mean percentages of the major constituents (192 samples) were: total solids, 20·0%: crude protein, 5·7%: lactose, 4·7%; fat, 8·6% and ash, 0·89%.

2. There were differences in milk composition between sows and between successive lactations, but these were only appreciable for milk fat percentage between sows.

3. A highly significant negative correlation ($P < 0{\cdot}001$; $r = -0{\cdot}62$) was found to exist between the percentages of protein and lactose in the milk, quite apart from the general trend for protein content to fall with advance in lactation. Possible reasons for this are discussed.

I wish to thank Mr I. MacDonald of the Statistics Department, the Rowett Research Institute, for statistical analysis of the data, and Mr J. Munro for analysing the milks.

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OBSERVATIONS BY ANOPTRAL CONTRAST MICROSCOPY OF CASEIN IN MILK, CONDENSED MILK AND MILK POWDER

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(With 1 plate)

Recently use has been made of fluorescence microscopy in the examination of different physico-chemical forms of casein in milk, curd and cheese, and certain advantages of this technique as compared with electron microscopy have been pointed out (1). However, some fluorescent stains, despite relatively high dilution, may affect the stability of the delicate protein system in the milk. In addition, the fluorochroming ability of fluorescent stains is variable, depending upon the degree of purity, pH, the presence of various reagents (quenching substances), concentration, temperature, etc.

Phase-contrast microscopy should be free from these disadvantages, and studies have therefore been undertaken of its application to the observation of the ultra-microstructure of dairy products. According to Cosslett (2) the contrast scheme from this method of optical microscopy is basically identical with that of an electron image, so that at the same magnification (e.g. $\times 1500$) it is often difficult to distinguish one from the other, especially if the phase-contrast 'halos' are faint. Barer (3) maintains that in certain cases of high contrast, particles as small as 50 mμ in diameter may be rendered visible in phase contrast. A drawback of phase contrast as applied to milk is that it does not enable large casein particles to be distinguished from small fat globules, for example in homogenized milk products. However, the casein particles and casein coagulation forms can be observed in their genuine environment and various reagents can be applied to them.

A new modification of phase contrast, the *anoptral contrast* by Wilska-Reichert, is claimed to increase appreciably the contrast and resolving power, enabling discernment of very fine structural details—membranes, fibrils, bacterial flagellae, etc. (4). This is achieved by using a special absorbing material for phase rings and wider annular apertures than those usual in phase-contrast microscopes. The image obtained in anoptral contrast is to some extent similar to that obtained with 'negative' phase contrast.

Experimental method

Wilska-Reichert's anoptral contrast outfit used in this work comprised three special objectives ($\times 20$, $\times 45$ and $\times 100$), an anoptral condenser and an auxiliary microscope for adjustment. It was fitted to a Cooke, Troughton and Simms research microscope, the light source being a high-power filament lamp of the same manufacturer. To obtain contrast-rich pictures of bright particles on a warmly tinted brownish background, careful adjustment is required of the distance between the lamp condenser and the light

globe, the position of the mirror, the distance between the anoptral condenser and the slide, and the position of the condenser in the horizontal plane. Slides and cover glasses must be free from imperfections and the specimens should be thin.

RESULTS

Milk

The fat globules above 1 μ in diameter suspended in milk are surrounded by a 'halo' which renders them unsuitable for investigation by the anoptral contrast. However, when the fat globules are embedded in liquid milk fat, as happens in the 'pools' of coalesced fat, no 'halos' are present on their periphery and fine details in their structure, very fine crystals and folded material, become visible. The casein particles appear as minute, sharply outlined, yellowish white, almost spherical (perhaps slightly flattened) bodies in vivid Brownian movement on a brownish background. When slightly out of focus the particles appear dark-violet.

Particles as small as 0·1–0·2 μ in diameter can be perceived. As in the fluorescence microscope (1) the particles at high magnification do not appear very dense, suggesting a rather loose aggregation of casein molecules. In anoptral contrast the casein particles are much more conspicuous than in the conventional phase contrast (cf. the photomicrographs of Mohr, Mohr & Drachenfels (5)) and many more of them show up than in the fluorescence microscope. In fresh milk the casein particles show no aggregation.

In skim-milk from an open separator there occur occasionally very thin, folded lamellae, apparently representing remnants of collapsed foam. Compared with the photomicrographs of different membranes given by Wilska (4), the thickness of these foam lamellae may be roughly estimated at 10 mμ. The basic material of the film, which does not show any structural details, contains a number of interspersed casein particles. It might be that this film consists of a 'blanket' of interwoven surface-denatured unfolded casein molecules. Foam lamellae are likely to form in the manufacture of the newly developed foam-dried milk powder and their study may be important in relation to the physical structure and dispersing properties of this product.

The picture of acid and rennet clotting in anoptral contrast was similar to that in the fluorescence microscope (1). It was observed that whereas the rennet clot from undiluted skim-milk showed only some few short micro-fibres, a pronounced formation of nodulated micro-fibres—sometimes branched, sometimes forming a network—took place on renneting skim-milk diluted 10 to 20 times with a 0·1% solution of calcium chloride (Pl. 1*a*). This is in agreement with the ultra-microscopic observations of Hekma (6), who likened the network of nodulated fibres to a cobweb with dew-drops attached. Hekma (7) points out that the network is very similar to that of blood fibrin. In spite of appreciable dilution of milk the clot in test tubes was coherent; it exhibited pronounced syneresis. In the micro-fibres, which were most readily discernible in smaller fragments of the clot, on the edges of large fragments, or overspanning holes in the clot, the casein particles were joined together by means of thin threads (about 0·1 μ wide). The phenomenon suggests a partial unfolding of casein molecules with the formation of 'tails' on the particles, which subsequently link them—a possibility supported by the electron microscopic observations of rennet clotting by Baud, Morard & Pernoux (8).

An excellent picture of nodulated fibres was also obtained in the fluorescence microscope by using thioflavine T as fluorochrome.

The casein micro-fibres in cheddared curd and in microsections of curd and cheese spread on water were smooth (1), which may indicate an advancing uncoiling and adlineation of casein molecules in the course of the further treatment of rennet clot.

Condensed milk

In some brands of evaporated (unsweetened condensed) milk (diluted 1:1) the casein particles overwhelmingly occurred singly, while in other brands there were quite numerous flocks consisting of loosely aggregated particles. In sweetened condensed milk (diluted 1:2) almost all the casein particles were single (Pl. 1*b*). Generally, the casein particles in evaporated milk appeared somewhat larger than in sweetened condensed milk, presumably due to the coalescing effect of homogenization and sterilization. The electron micrographs of Hostettler & Imhof (9) show a distinct agglomeration of casein particles in homogenized milk, with the formation of secondary particles of ragged outlines. During sterilization the particles will probably melt and take a spherical shape.

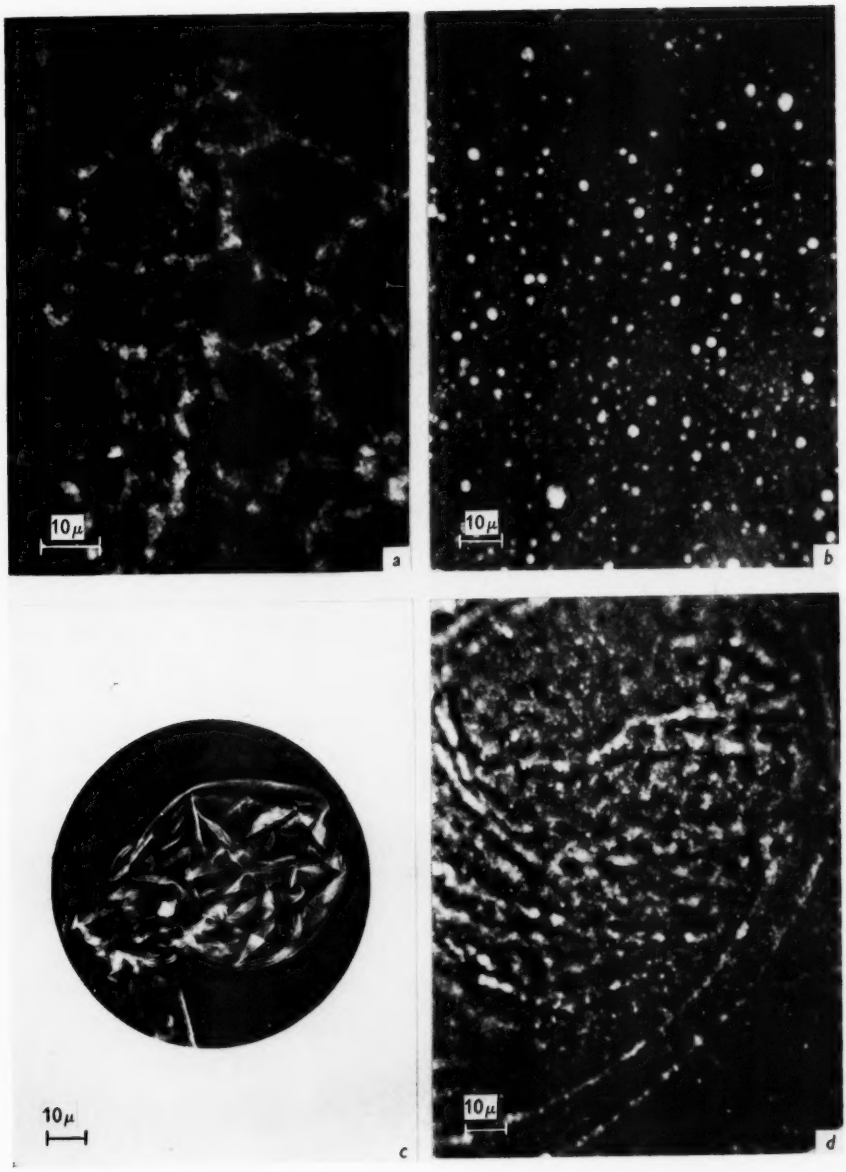
The electron microscopic observations of Hostettler & Imhof (10) and of Huth (11) tended to confirm the observed differences between evaporated and sweetened condensed milk, the latter worker recording mean diameters of casein particles of $0{\cdot}1\,\mu$ in pasteurized skim-milk, $0{\cdot}16\,\mu$ in sweetened condensed and $0{\cdot}39\,\mu$ in evaporated milk. The coagulation forms in the electron micrographs appear appreciably denser in comparison with the loosely aggregated flocks observed in anoptral contrast. Apparently some fusion of flocks has occurred in the former, owing to the temperature rise in the electron beam.

Milk powder

Anoptral contrast allows observation of the dispersion state of casein in reconstituted milk, which in turn depends upon the method of manufacture and storage conditions of the milk powder. Milk reconstituted at 20° C., according to Howat, Smith, Waite & Wright (12), from roller-dried powders contained, apart from quite a number of single casein particles and small loose aggregates, appreciable quantities of swollen powder particles and their debris. With spray-dried powders the single particles and small aggregates were much more numerous, whereas the amount of swollen powder particles was less—a picture which is in accord with the better dispersibility of this kind of powder. On reconstituting at 50° C. only very few powder particles were left, and almost all microscopically-visible casein occurred in single particles. The agitation during reconstitution of spray-dried skim-milk or of the reconstituted product usually provokes pronounced frothing. On collapsing of the froth thin folded proteinaceous membranes, similar to those occurring in skim-milk from an open separator (cf. above), could be encountered in the milk (Pl. 1*c*). The walls of non-collapsed air bubbles inside the reconstituted milk contained single casein particles, as well as aggregates of particles of irregular and linear shape (Pl. 1*d*).

SUMMARY

Anoptral contrast, a modification of phase-contrast microscopy, appears to be suitable, particularly perhaps when used in conjunction with fluorescence microscopy, for studying casein particles above $0{\cdot}1$–$0{\cdot}2\,\mu$ in diameter, and different aggregation and coagulation



KING—Anoptral contrast microscopy of casein

(*Facing p.* 142)

forms of casein, which may comprise irregular flocks of aggregated particles, thin membranes and micro-fibres, nodulated or smooth.

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EXPLANATION OF PLATE 1

a. Nodulated micro-fibres of casein in the rennet clot from skim-milk diluted (1:10) with a 0·1% solution of calcium chloride ('Hekma-phenomenon'). Anoptral contrast.
b. Fat globules and casein particles in sweetened condensed milk. Anoptral contrast.
c. Lamella of collapsed foam in reconstituted spray-dried skim-milk. Anoptral contrast.
d. Casein particles and aggregates in the wall of a non-collapsed air bubble inside the reconstituted skim milk. Anoptral contrast.

PHYSICAL CHANGES IN MILK CAUSED BY THE ACTION OF RENNET

V. EFFECTS OF VARYING CONCENTRATIONS OF MILK POWDER AND OF LOW-TEMPERATURE PRETREATMENT

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(With 3 Figures)

In the previous papers in this series, which will be referred to by Roman numerals, Scott Blair & Burnett[1,2,3] described a method for measuring the increasing rigidity of renneted milk and the effects of various milk treatments on the rigidity moduli and viscosities. The following nomenclature is used: overall modulus G, 'immediate' modulus G_1, 'slow' modulus G_2, external creep viscosity η_1 and internal damping viscosity η_2. All these parameters give good straight lines during most of the setting time (t) when plotted against log t. Their slopes ($dG/d(\log t)$ or $\dot{G}t$, etc.) and their intercepts on extrapolation to the time axis (I), give convenient measures, respectively, of the (logarithmic) rates of setting of the gels and of what may be called the 'induction periods', slightly longer than the observed coagulation times (t_c).

The present paper describes experiments on the same reconstituted fat-free dried milk as before, under standardized conditions of rennet concentration, temperature, amounts of added calcium chloride and lactic acid but varied concentrations of milk powder and lactose.

In a second series of experiments, the rennet was allowed to act on the milk at normal concentration for 1½ hr. at a temperature of about 2° C., after which the milk was warmed as quickly as possible to a series of pre-selected setting temperatures, the rates of setting being measured as before.

Experimental

Experiment 1. *Effects of variations in milk powder concentration*

Under standard conditions, 9 g. of milk powder were made up with 1 ml. of 2% rennet, 1 ml. of 2% lactic acid and 15 mg. of Ca as calcium chloride, to 100 ml. In the present experiment, smaller and larger quantities of powder were also used, keeping all other conditions unchanged. Since about 52% of the powder was (hydrated) lactose as against about 28% casein, it seemed also worth while to study the effects of adding 1 and 2 g. of lactose to a standard 9 g. sample to find out whether these quite large additions of lactose appreciably affected the setting properties. The results are listed in Table 1 in which the following additional symbols are used, as in the earlier papers; λ is the proportion of elastic recovery which is 'delayed' as distinct from 'immediate'; $t_{\text{ret.}}$ is retarda-

tion time defined as η_2/G_2; $t_{rel.}$ is relaxation time defined as η_1/G_1; N is springiness number defined as $t_{rel.}/t_{ret.}$.

These results may be summarized as follows: The values of $\dot{G}t$, $\dot{G}_1t$ and $\dot{G}_2t$ increase progressively with increasing concentration. The logarithms of their values are plotted against logarithm of concentration in Fig. 1 and it is seen that all these parameters vary approximately as a power of concentration, the exponent being about 2·5.

Table 1. *Effects of variations in milk powder concentration and of added lactose on setting parameters*

Parameter		Concentration, g./100 ml.					9 g./100 ml. + lactose (g.)	
		5	7	9	11	13	1	2
min.	t_c	4·5	9·0	10·0	14·5	16·5	11·0	10·5
	I	7·5	11·0	15·0	19·0	21·0	16·0	15·0
dynes/cm.^{2}	$\dot{G}t$	58	115	230	365	530	210	225
	$\dot{G}_1t$	112	210	450	680	975	350	425
	$\dot{G}_2t$	120	250	530	775	1165	520	505
poises × 10^{-3}	$\dot{\eta}_1t$	7·50	12·50	32·0	40·0	64·5	25·9	28·5
	$\dot{\eta}_2t$	4·00	8·50	17·0	24·5	34·0	18·3	17·5
	λ	0·46	0·45	0·45	0·46	0·45	0·42	0·45
sec.	$t_{ret.}$	30·0	30·3	30·9	37·9	28·5	32·6	34·7
	$t_{rel.}$	72·0*	70·5	65·5	52·0	52·2	74·3	64·0†
N		2·32	2·18	2·18	1·62	1·85	2·33	1·84

* Value falling with time. † Value rising with time.

The values of $\dot{\eta}_1t$ and $\dot{\eta}_2t$ likewise increase as a power of the concentration, as shown in Fig. 2; but the power is here about 2·3.

The values of t_c and of I increase nearly linearly with increasing concentration, I being some 20% greater than t_c throughout.

The values of $t_{rel.}$ and of N tend to fall as concentration increases, while those of λ and of $t_{ret.}$ remain almost unchanged. Adding quite large amounts of lactose, so as to increase the lactose content by some 21 and 42% respectively, makes little difference to the values of t_c or I. There appears to be a slight reduction in $\dot{G}t$ and hence in $\dot{G}_1t$ and $\dot{G}_2t$, and perhaps also a slight reduction in $\dot{\eta}_1t$ with little or no change in $\dot{\eta}_2t$. The creep viscosity, $\dot{\eta}_1t$, is the least accurately determined of the Burgers parameters. There are no appreciable effects on $t_{rel.}$ and N, but there is some tendency for $t_{ret.}$ to increase. This last observation is interesting, since $t_{ret.}$ has been found to be remarkably unaffected by all other changes in conditions which have so far been tried.

It is clear from the lactose experiments that the main effects, when the overall concentration of powder is changed, are due to changes in the protein concentration. The part played by soluble proteins has not yet been investigated so that it is not possible to ascribe these effects entirely to the casein.

The results show an interesting contrast to those quoted in IV, when the amount of rennet was varied. Increasing rennet, it will be remembered, *diminished* t_c and I, as well as $\dot{G}t$, whereas increasing milk concentration *increases* t_c and I while diminishing $\dot{G}t$ and similar parameters. We conclude from this that observations of t_c alone will tell us little about the effects of any variant on the rate of setting of milk. This effect is not an artifact produced by the use of logarithmic setting rates; if G is plotted directly against t,

without logarithms, a series of curves is obtained which, though not lending themselves to quantitative description, clearly show the same effects qualitatively.

It will be remembered that, in changing rennet concentration, $\dot{G}t$ varies as a power of the concentration (see IV), but this power (3·0) is significantly greater than that relating milk concentration to the same parameter (2·5). In Fig. 1 the dotted line corresponds to a power of 3·0. The viscosities, especially η_1, being less accurately determined, the difference in the slopes in Figs. 1 and 2 cannot be regarded as certainly significant.

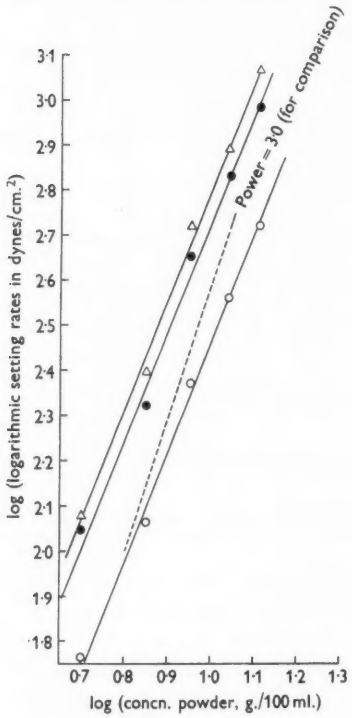

Fig. 1

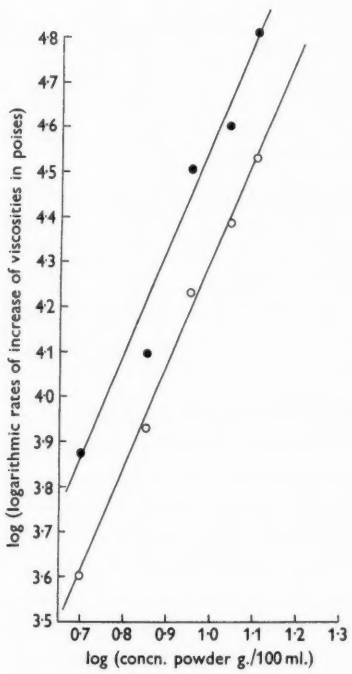

Fig. 2

Fig. 1. Milk powder concentration and elastic constants: ○—○, log($\dot{G}t$); ●—●, log ($\dot{G}_1 t$); △—△, log ($\dot{G}_2 t$).

Fig. 2. Milk powder concentration and viscous constants. ●—●, log ($\dot{\eta}_1 t$); ○—○, log ($\dot{\eta}_2 t$); slope of lines 2·3.

Experiment 2. Effects of separating two stages of rennet action

Although earlier qualitative observations were recorded, Berridge (4) was the first to study quantitatively the effects of clotting milk in two stages. Rennet is allowed to react with the milk for some time at a temperature just above the freezing-point, after which the temperature is raised as quickly as possible to the normal range, when clotting very soon starts.

In the experiment here described, comparison was made between the G/log t curves for the standard powdered milk with the usual additions by the normal process (N.) and a separated action process (S.A.) at the same setting temperatures as were used in IV, excepting only the highest temperature (41° C.). The renneted milk was first held for

$1\frac{1}{2}$ hr. at about 2° C. and then poured through a heat exchange unit straight into the U-tube of the gelometer, the temperature being thus raised to the pre-selected value (θ° C.) in about 6 sec. The first reading was generally taken 1 min. after the tube was filled, subsequent readings being taken as in the normal experiment.

The S.A. experiment is not very easy to do and the scatter of the points on the $G/\log t$ curves is higher than in the N. experiment. In these experiments, the stresses are always applied in alternate directions and the scatter would appear to be due mainly to a difference in modulus when the curd is stressed in one or other direction. In the normal process of renneting, this phenomenon, called 'divergence', is observed only when the curd has been stressed when very soft (see III and Scott Blair & Burnett(5)). In the S.A. process, it appears much more frequently and is often impossible to prevent. At 41° C., it was not possible to get a normal curd by this method and the very rapid onset of some kind of syneresis made the test impossible. At 38° C. and, to a lesser extent, at 35° C., the reproducibility was not as good as in normal experiments. The figures quoted below were the best obtainable and there seems little doubt of the trends in the parameters. At temperatures of 32° C. or less, in spite of the scatter already mentioned, the reproducibility of the curves was reasonably satisfactory.

The heat exchange unit consists of a helical metal pipe surrounded by stirred heated water, the temperature of which was adjusted by trial and error to give the required temperature to the outflowing milk. Flow in this pipe was certainly turbulent and, on theoretical grounds, this method of heating appeared to be satisfactory at least for preliminary experiments. However, should this S.A. process prove to have a practical application, it would, no doubt, be worth while to try to improve this rather difficult process of quick heating of these small samples, so as to get better reproducibility.

In the preliminary experiments here recorded, it was found that some time elapses before the points in the S.A. experiment fall on a straight line, plotting $G/\log t$. At the lowest two temperatures G and t give very nearly straight lines on a direct plot over quite a wide range. If the coagulation time (t_c) for the corresponding N.-run is added to the time after heating, before taking the logarithm, this 'tailing off' is reduced to normal dimensions. Further, if in an N.-run, t_c is subtracted from the time after renneting before taking logarithms, curves very similar to the S.A. curves as originally plotted, with marked 'tailing-off', are obtained. This means that the linearity of the $G/\log t$ plot depends on taking the setting process as a whole, an important point which will be discussed later.

An experiment was done at 32° C. in which all the Burgers parameters were compared as between S.A. and N. However, since no important differences were found, apart from those shown in the simpler experiment, the latter only was done at the other temperatures. The results are given in Table 2. It would not be quite fair to compare the values of

Table 2. *A comparison of normal* (N.) *and two-stage* (S.A.) *clotting of milk by rennet at different temperatures* (θ)

θ° C.	21		25		29		32		35		38	
	N.	S.A.	N.	S.A.	N.	S.A.	N.	S.A.	N.	S.A.	N.	S.A.
I (min.)	46	51	22	34	17	15	15	9	9	6	9	7*
$I+t_c$	—	86	—	50	—	27	—	19	—	13	—	13·5*
$\dot{G}t$ (dynes/cm.²)	105	93	132	186	214	194	230	196	186	120	175	118

* Readings of doubtful reliability.

I for N. and S.A. experiments without qualification, since in the N. experiment, the intercept includes the time needed for the process which, in the S.A. experiment, takes place in the cold before the starting time is reached. To over-simplify, if the action of rennet consisted of no more than two processes, one which can take place in the cold involving no rigidity and the other consisting of a steady increase in G at a temperature θ following a linear $G/\log t$ plot, then the N. intercepts should be compared with the sum of the S.A. intercepts and the N. coagulation times. These figures are also given in the table.

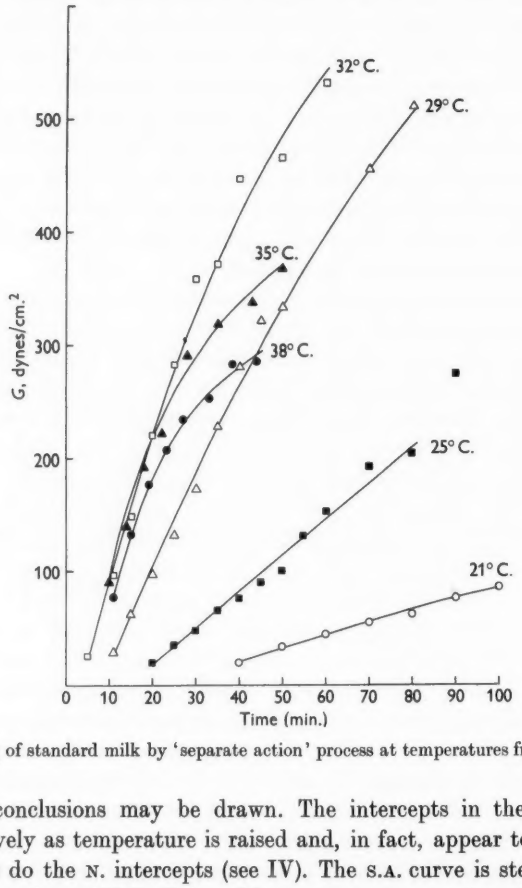

Fig. 3. Setting of standard milk by 'separate action' process at temperatures from 21–38° C.

The following conclusions may be drawn. The intercepts in the S.A. experiment decrease progressively as temperature is raised and, in fact, appear to follow the same exponential law as do the N. intercepts (see IV). The S.A. curve is steeper than the N. curve, crossing it at about 27° C. Below this temperature, even ignoring altogether the induction period inherent in the N. but not in the S.A. experiments, the latter takes longer to establish the normal process of gelation. The logarithmic setting rates ($\dot{G}t$) pass through maxima at about 32° C. with both processes. Thus, even when the part of the reaction which can take place in the cold is eliminated, it is clear that high temperatures reduce the speed of setting. This is seen more clearly, perhaps, if the data are plotted on a linear time scale (Fig. 3). This plot also shows the advantages, for purposes of tabulation, of using the log t plot, since the data cannot be described quantitatively in terms of linear time. The phenomenon of 'divergence' is clearly shown, especially at 32° C.

DISCUSSION

It will be recalled from III that the remarkable linearity of normal setting curves when G is plotted against log t reflects the approximate linearity of a more meaningful plot, namely log $[(G_\infty - G)/G_\infty]$ against t, where G_∞ is the rigidity when almost all the casein has set. This means that, when G is linear with log t, it is true to say that the rate of setting of the curd at time t is approximately proportional to the amount of casein still unset at that time. The reason why this more complex plot is not made as a routine for quantitative description is that the selection of G_∞ is arbitrary. The fact that G increases linearly with log t with such precision for so long makes the determination of any kind of asymptote impossible. The later stages of the setting may, however, be very accurately described in terms of the two parameters I and $\dot{G}t$. Moreover, since, when various factors are altered, these parameters vary quite independently, sometimes changing in the same and sometimes in opposite directions, it is clear that I alone, or 'coagulation time', which differs very little from it, is a quite inadequate criterion for describing setting.

Much has been written about the nature of the setting process. Our own experiments confirm that (i) part of the process, not involving any appreciable rigidity, can take place in the cold; (ii) when the temperature is then quickly raised, rigidity begins to develop, but not, at first, very rapidly. At lower temperatures, at least, the delay in reaching a 'first order' setting-rate, as measured by I, is greater than that when the first part of the process is not carried out previously in the cold; (iii) those processes which occur after the first stage has taken place in the cold, are probably as much delayed by high temperatures as are those which take place under normal conditions of renneting.

Meanwhile, we have been unable to offer any information about the very early stages of clotting. We do not yet know what changes, if any, in viscosity take place (*a*) with renneted milk in the cold, and (*b*) in the normal process before G can be measured in the gelometer, nor what laws govern the process from the time when rigidity first appears until it is sufficiently established to be measured in the gelometer.

Experiments are in progress which, it is hoped, will answer these questions. Until this work is completed, we prefer to refrain from any detailed discussion on the implications of our experimental results for the current theories of clotting.

SUMMARY

1. When the concentration of fat-free milk-powder is increased, other factors being kept constant, the elastic and viscous setting-parameters of the curd also progressively increase, being related to the concentration by power-equations. The intercepts, which correspond closely to 'coagulation times', likewise increase, but nearly linearly with concentration

2. These effects depend mainly on protein concentration. Quite large quantities of added lactose make little difference to the parameters.

3. The slopes of the logarithmic setting curves vary quite independently of their intercepts, so that 'coagulation times' provide a very incomplete indication of the rate of setting of curd.

4. A comparison of setting data obtained in the ordinary way at a series of temperatures with those from experiments in which the rennet is first allowed to act on the milk

at near freezing-point, show that, in the latter case, there is considerable delay before the normal equation of setting is established in the curd. It is also clear that high temperatures are detrimental to this process of setting, just as they are when the usual procedure is followed.

5. The general law that the rate of setting is, at any time, proportional to the amount of still unset casein, is established much sooner if the time zero is defined in such a way as to take all the processes involved together. If an 'induction period' is subtracted, either by letting the rennet first act in the cold, or by taking the time zero in the normal process at the coagulation, and not the renneting, time, a much longer time elapses before the curves become linear.

We are indebted to many colleagues at the N.I.R.D. for helpful discussions, especially Dr S. J. Rowland and Dr R. Aschaffenburg.

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A PROPOSED METHOD FOR THE RAPID DETERMINATION OF LACTOSE IN SEPARATED MILK AND CONDENSED WHEY BY INFRA-RED ABSORPTION

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(With 3 Figures)

The analysis of dairy products by infra-red spectroscopy presents a number of special difficulties due to the aqueous medium and to the non-homogeneous nature of the materials presented for study(1). A number of water-resistant optical materials are now available for use as cell-windows(2), and Potts & Wright(3) have shown that quantitative analyses can be carried out successfully with aqueous solutions. Although the intense absorption of water obscures much of the infra-red region, it is relatively weak near 10μ, where lactose shows strong absorption bands.

The fat globules in whole milk cause considerable scattering, since their diameters are not much less than the wavelengths in the 10μ region. Separated milk, however, provides a more favourable system for infra-red analysis, since it contains casein micelles with diameters of the order of $0{\cdot}1\mu$, i.e. about a hundredth of the wavelength at 10μ. It is therefore expected that the effects due to radiation scattering will be relatively small for separated milk and even less for whey, where the casein is no longer present.

During the condensing of separated milk and whey, it is important to determine rapidly the degree of concentration achieved. This determination is usually carried out by methods based upon specific gravity, viscosity, refractive index and, in the case of whey concentrates, on titratable acidity. Recently, methods based upon electrical conductivity and γ-ray absorption have been proposed(4). Infra-red spectroscopy provides a further possible method and is readily adaptable to continuous recording.

Fig. 1 illustrates the absorption spectra of samples of separated milk and whey examined in a cell of path-length 62μ, a cell of the same path-length containing water being placed in the comparison beam of the spectrometer. From the spectrum of the casein suspension also shown in Fig. 1, it can be seen that the relative contribution of the protein to the total absorption is less at the lower frequency bands where the absorption is due largely to the lactose. The most convenient band for the determination of lactose in separated milk is therefore the shoulder at 1040 $cm.^{-1}$.

In condensed whey samples, the lactose absorption at 1040 $cm.^{-1}$ becomes so intense that very short path-length cells must be used to ensure optimum absorbance values. Since the casein content is considerably lower in the whey samples, it is possible to use the relatively weaker bands at 1117 and 1155 cm^{-1} for the analysis of moderately concentrated whey samples, and thus avoid the use of the very short path-length cells.

EXPERIMENTAL

A Grubb Parsons S3A double beam spectrometer fitted with a rocksalt prism was used for examination of the spectra. Samples were examined in fixed path-length cells manufactured by the Research and Industrial Instruments Company and fitted with barium fluoride windows. A matched pair of cells with path-lengths 62μ was used for the study of separated milk samples and a single cell of nominal path-length 10μ was used for the

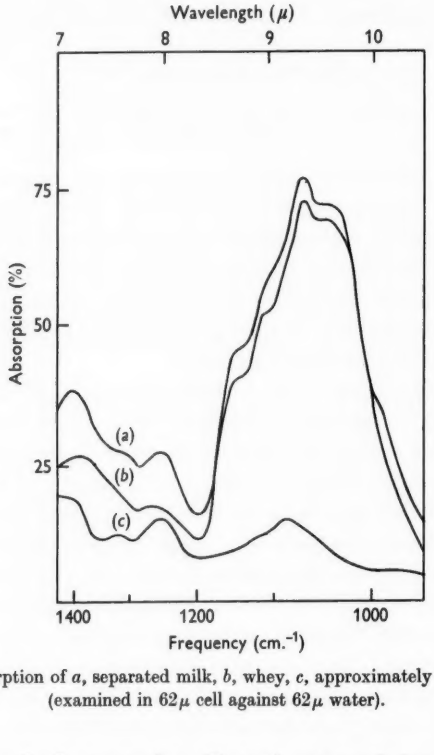

Fig. 1. Infra-red absorption of *a*, separated milk, *b*, whey, *c*, approximately 4% casein suspension (examined in 62μ cell against 62μ water).

analysis of the condensed whey samples. Since the transmission of a 62μ thick water film is very low in the 9μ region, it was necessary to use wide instrumental slit-widths of 1 mm. Narrower slit-widths of 0·5 mm. could, however, be employed with the 10μ path-length cell. These wide slit-widths did not result in large deviations from the Beer's Law relationship, as demonstrated by the linear relationships shown in Figs. 2 and 3. When the 10μ cell was in use, a grid was placed in the comparison beam to displace the zero absorption curve close to the chart zero line. An F-centred KCl crystal was used as a filter to eliminate stray radiation from wavelengths greater than 2μ, although the intense absorption of the water itself eliminated much of the shorter wavelength stray radiation.

Separated milk and whey concentrations were carried out in a laboratory rotary film vacuum evaporator, to avoid difficulties associated with foaming. Pressures of about 40 mm. Hg were used, corresponding to evaporation temperatures in the 30°–40° C. range.

The lactose concentrations were determined by the Fehling's solution titration method (5), standardized against lactose solutions of known concentrations. The protein was removed prior to titration by precipitation with lead acetate solution, excess lead acetate being precipitated by adding a saturated solution of potassium oxalate (6). All lactose concentrations have been expressed as g. anhydrous lactose per 100 g. liquid.

The total solids contents were determined by heating 1 ml. samples of whey or separated milk in aluminium dishes to constant weight at 100°–105° C. After allowing a small constant correction of 0·9% for the salt content, the differences between the total solids and lactose contents of the separated milk samples were used as a measure of the protein concentrations in the different separated milk samples.

For the attempted calibration experiments, casein was isolated as a gel from separated milk with the aid of a Spinco Model L Ultracentrifuge ($g \sim 50{,}000$). The gel was redispersed in water and centrifuged three further times before freeze-drying. The spectrum of the casein suspension shown in Fig. 1 was obtained from a sample of the freeze-dried casein redispersed in water.

RESULTS

Separated milk

To test the accuracy of the method with separated milk samples of differing composition, milk samples were collected from cows in the Institute herd, selected to include as wide as possible a range of differing lactose and protein contents. For this series of analyses the 62 μ path-length cells were used to ensure a convenient range of absorbancies (or optical densities), and the results are shown in Fig. 2.

From the spectra shown in Fig. 1, it can be calculated that about 90% of the absorbance at 1040 cm.$^{-1}$ in the separated milk spectrum is due to the lactose, the remainder being due to absorption and scatter by the proteins. Changes in protein content will therefore have only a second-order effect upon the absorption at 1040 cm.$^{-1}$, and, although the calculated protein contents of the different separated milk samples varied between 2·9 and 3·7%, no direct correlation could be found between the protein content and the magnitude of the deviations of the points in Fig. 2. The maximum deviations were found to correspond to $\pm 1{\cdot}5$% of the lactose concentration values, this limit being set by the normal accuracy of the photometric system of the spectrometer.

Condensed whey

Whey samples were supplied by the Institute Dairy and were centrifuged for a few minutes at a low speed prior to concentration, in order to remove residual fat. Apart from effects due to absorption and scattering, the presence of fat made difficult the filling of the short path-length absorption cells without the introduction of air bubbles.

Fig. 3 shows some of the results obtained with the 10 μ path-length cell. Similar relationships were found for the 1117 and 1072 cm.$^{-1}$ peaks, although these have not been illustrated. From Fig. 3 it can be seen that this method permits the analysis of condensed whey samples containing up to at least 35% lactose and that the maximum error was found to be about ± 8% of the lactose value. The points shown in Fig. 3 were obtained from the analyses of three samples of condensed whey prepared on different occasions during the course of a month.

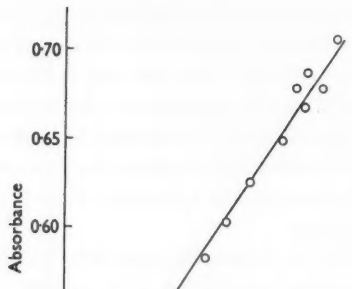

Fig. 2

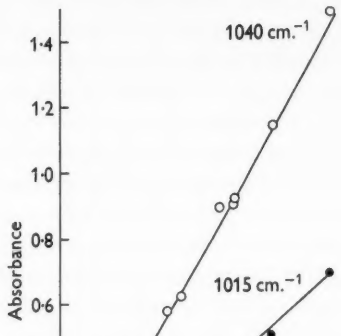

Fig. 3

Fig. 2. Relationship between absorbance of separated milk and lactose concentration at 1040 cm.$^{-1}$ (examined in 62μ cell against 62μ water).

Fig. 3. Relationship between absorbance of condensed whey and lactose concentration at 1040 cm.$^{-1}$ and 1155 cm.$^{-1}$ (examined in $\sim$10μ cell).

Condensed separated milk

Attempts were made to study condensed separated milk, but owing to difficulties in cell filling and cleaning, it was found impossible to examine a large number of condensed separated milk samples. The presence of the casein made necessary frequent cleaning of the cell with dilute alkali in order to prevent the formation of air bubbles and to remove solid deposits from the cell windows. During this cleaning process, the barium fluoride windows appeared to be attacked slowly, so that the cell path-length increased. These difficulties did not appear with condensed whey or with untreated separated milk when the latter was used in the 62μ path-length cells.

The preliminary results obtained indicated that in the case of the more concentrated samples with lactose concentrations of the order of 15%, the contribution of the protein to the total absorbance by both absorption and scattering amounted to more than 40%. Some of this increase may well have been caused by deposits being built up on the cell window whilst the warm solution remained in the absorption cell.

DISCUSSION

From the data shown in Figs. 2 and 3, it can be seen that the direct measurement of the absorbance at 1040 cm.$^{-1}$ provides the basis of a rapid method for the analysis of the lactose contents of both separated milk and condensed whey. It was hoped originally to obtain a direct calibration from absorbance measurements on casein suspensions and on lactose solutions separately, so that the exact slope of the calibration graph could be

calculated, but this was found to be impossible owing to difficulties associated with the preparation of reproducible casein suspensions.

For the analyses of the condensed whey samples, the accuracy was found to be only about $\pm 8\%$, but it is considered that this could be improved by the use of a continuous-flow type absorption cell fixed permanently in position. The main source of error in the condensed whey analyses arose from the need to remove the cell holder completely from time to time so that the spectrometer could be used for other work. Accuracy of cell location becomes particularly important for short path-length cells.

A further difficulty in the use of such cells arises from the need to ensure that small air bubbles are not present in the illuminated area of the cell. This difficulty should also be removed by the use of a continuous flow technique, although the addition of a trace of Teepol was found to assist the filling of the cells.

The infra-red analysis of whey concentrates could be carried out with a fixed wavelength single-beam spectrometer or even with a simplified filter instrument when suitable filters become available. Unlike the methods based upon viscosity or specific gravity, accurate temperature control is not required for the infra-red method, since absorption intensities and cell path-length are much less dependent upon temperature than viscosity or specific gravity.

The choice of cell path-length is governed to some extent by the concentration range of interest. Provided sufficient energy is available, it should be possible to use longer path-length cells, e.g. 20μ, 40μ, together with a differential technique for the examination of whey concentrates. Apart from increasing the accuracy, longer path-length cells would be easier to clean and handle. Owing to the intense water absorption, it is unlikely that cells much thicker than 62μ will be of value, since the transmission of a 62μ film of water is only about 3% in the 9μ region. For routine use, cells made from polythene film supported on rocksalt plates(7) may well prove more serviceable than the fragile barium fluoride windows used in the present investigation.

The major difficulties in the application of this technique to routine factory control are likely to centre round the absorption cell. To ensure a sufficiently rapid liquid flow through cells of such short path-lengths, considerable liquid pressure may be required and this possibly could lead to distortion of the cell, with consequent increase in optical path-length. It will also be essential to avoid the formation of deposits on the cell windows, and in practice it may be found necessary to employ a pair of cells so that one can be cleaned while the other is in use.

It is anticipated that this method may find wider application outside the field of dairying, particularly in the analysis of concentrated sugar solutions where difficulties due to proteins and fats are not experienced.

SUMMARY

It has been shown that the lactose concentration in separated milk can be determined rapidly with an accuracy of $\pm 1{\cdot}5\%$ by infra-red absorption methods, using wavelengths in the 10μ region.

The method can also be used to determine lactose in condensed whey and should prove applicable to continuous recording techniques.

The author is indebted to Dr S. J. Rowland for his advice and encouragement and also to Mr A. W. Wagstaff and Miss J. M. S. Kingston for assistance in carrying out the lactose determinations.

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LACTOBACILLI IN CHEDDAR CHEESE*

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(With 1 Figure)

Before the development of selective media for lactobacilli, it was almost impossible to determine the numbers of these organisms during the early stages of cheese ripening, when the starter streptococci were dominant. Early workers (1, 2) consequently assumed that the lactobacilli did not reach significant numbers until the streptococci had declined appreciably in numbers. This view is still expressed in recent text-books (3, 4). Recent British work (5) however, using a medium which inhibited growth of the starter streptococci, indicates that the lactobacilli grow slowly but steadily from the beginning of the ripening period. This has recently been confirmed by Naylor & Sharpe (6), who made intensive studies of one experimental cheese over a 6-month period.

Since 1954 the Dairy Technology Research Unit has been studying the enhancement of flavour in Cheddar cheese made from pasteurized milk. In each series of experiments, representative cheeses have been examined at intervals for the numbers of various groups of bacteria—lactobacilli, enterococci, coliforms, lipolytic and proteolytic—as well as the total viable count. Thus we have accumulated a considerable volume of data. In view of the recent report (7) of erratic fluctuations in counts of lactobacilli it was considered timely to report our quite different findings.

MATERIALS AND METHODS

In our experimental cheese-making, samples were taken (*a*) of the milk in the vat before adding the starter but after other special inocula had been added, and (*b*) of the cheese after approximately 2–5 days, 2 weeks, 3, 6 and 12 months. The cheeses were customarily ripened at 58° F. for 2 weeks, then at 40° F. for 1 year or longer. Cheese samples of about 10 g. were taken from the 10 lb. cheese with a trier, discarding the surface portion. After grinding with a pestle and mortar, 5 g. were weighed out and added to 95 ml. of 2% sodium citrate solution tempered at 45° C. After shaking to dissolve and suspend the cheese, further dilutions were made using buffered distilled water (8) and plates poured from appropriate dilutions with Rogosa's (9) medium. Counts were made after 5 days' incubation at 32° C.

The types of milk used included (A) Central Experimental Farm (C.E.F.) raw milk, a low count milk produced under good sanitary conditions; (B) the same milk pasteurized to 161° F. for 16 sec., cooled to 40° F., and held overnight; (C) raw milk brought in from a cheese factory; (D) the same milk pasteurized; (E) as D but inoculated with selected strains of lactobacilli at the start of cheesemaking.

* Contribution no. 31 from the Dairy Technology Research Institute.

RESULTS AND DISCUSSION

The development of lactobacilli has been determined in 28 cheeses made from the five classes of milk in the following numbers, A–2, B–4, C–10, D–6 and E–6. (In addition, determinations were made on ten other vats which received other types of inocula.) In order to obtain representative growth curves, log. plate counts were graphed for all cheese in each class, then a typical curve selected. These representative curves are shown in Fig. 1, while the log. average counts for all cheeses in each class appear in Table 1.

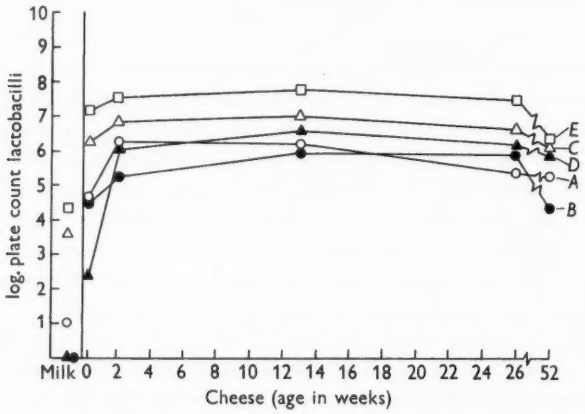

Fig. 1. Representative growth curves (log. plate counts) of lactobacilli in Cheddar cheese made from various types of milk. A (○—○), C.E.F. raw; B (●—●), C.E.F. pasteurized; C (△—△), factory raw; D (▲—▲), factory pasteurized; E (□—□,) factory pasteurized, factory pasteurised.

It will be noted that during the first few days the counts of lactobacilli had usually increased several hundredfold over those in the milk. It is also worth noting that in Fig. 1, the count level at each sampling period was generally correlated with the initial lactobacillus count in the milk. This is also evident in Table 1, although the cheeses from the pasteurized C.E.F. milk (B) attained a surprisingly high level of count at 3 months and beyond. While an occasional cheese displayed minor fluctuations in count level, the common picture showed a smooth curve which rose sharply during the first 2 weeks, then more slowly to the third or sixth month. By the sixth month the curve had sometimes declined a little, while at the end of 1 year the drop was more pronounced. However, counts as high as 29×10^6/g. were encountered after 1 year, while 18 of 38 cheeses gave counts of one million or more per g. at this time.

Mabbitt & Zielinska (5) reported a fairly steady increase in numbers of lactobacilli over a period of about 130 days. (The temperature at which the cheese was cured was not stated.) Naylor & Sharpe (6) found no appreciable increase after 50 days, when the curve flattened out. Our results based on a much larger number of samples representing a wider range of flora, agree better with those of the latter workers. However, our curves show a much steeper climb to a plateau during the first 2 weeks of ripening, with surprisingly high counts after 2–5 days. The maximum count recorded, 8×10^8/g., was obtained on a cheese made from factory raw milk (C) after 3 months' ripening.

Table 1. *Logarithmic average counts of lactobacilli in milk and cheese*

Class of milk	No. of samples		Milk		Cheese									
					2–5 days		2 weeks		3 months		6 months		12 months	
			Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
A	2	Log. count	1·90	1·266	3·64	1·478	5·37	1·223	5·68	0·672	5·100	0·283	4·750	0·636
		Count*	79	—	4,400	—	230,000	—	470,000	—	130,000	—	56,000	—
B	4	Log. count	—	—	3·042	1·078	4·562	1·066	6·152	0·242	6·742	0·641	5·567	1·533
		Count*	<1		1,100	—	37,000	—	1,400,000	—	5,500,000	—	370,000	—
C	10	Log. count	2·996	0·719	5·895	0·581	6·605	0·753	7·191	0·833	7·087†	0·564	6·169	0·646
		Count*	990	—	790,000	—	4,000,000	—	15,000,000	—	12,000,000	—	1,500,000	—
D	6	Log. count	—	—	3·615	0·664	5·580	0·452	6·39	0·5279	6·533‡	0·7617	5·594§	0·966
		Count*	<1	—	4,100	—	380,000	—	2,500,000	—	3,400,000	—	390,000	—
E	6	Log. count	3·836	0·411	6·930	0·798	7·285	0·229	7·713	0·694	7·790§	0·887	6·570	0·513
		Count*	6,900	—	8,500,000‖	—	19,000,000	—	52,000,000	—	62,000,000	—	3,700,000	—

* Mean counts derived from means of transformed data. † Seven samples. ‡ Three samples. § Five samples. ‖ Four samples.

The possible role of lactobacilli in the development of Cheddar cheese flavour has been studied by various workers (2, 9, 10, 11, 12). Some strains were reported to improve the flavour while others had the opposite effect. With a view to enhancing the degree of desirable flavour in Cheddar cheese made from pasteurized milk, we have used as inoculum a collection of 10 strains of lactobacilli isolated by us from high-scoring commercial raw-milk Cheddar samples. Milk cultures of these were added to the pasteurized milk at the start of cheesemaking, in numbers varying between 2300 and 21,000 per ml. Taste panel studies, using a ranking method (13), have invariably indicated that this treatment enhanced typical Cheddar flavour intensity, although not to the level attained by cheese made with factory raw milk. The following figures show the results of taste panel studies on the same vats of cheese examined after 6 months and 1 year, the lowest score indicating the highest flavour intensity:

	Flavour intensity points at	
Type of milk	6 months	1 year
Factory raw milk	26	18
Factory pasteurized milk	43	43
Factory pasteurized milk inoculated with lactobacilli	31	31

These findings suggest that one or more factors in addition to the lactobacilli are concerned in the development of Cheddar cheese flavour. Studies now in progress promise to throw some light here.

A relationship between the initial level of lactobacillus count and typical Cheddar flavour intensity has also been observed. The cheeses made from pasteurized milk (classes B and D) have always shown the lowest flavour intensity, while those made from factory raw milk (C) or the same milk pasteurized and inoculated with lactobacilli (E) have showed appreciably more flavour development. We have not attempted to determine the upper limit for the inoculum; Sherwood (11) reported best flavour results at a level of about 12,000/ml.

In view of the different effects of various strains of lactobacilli in cheese-making, attempts are currently being made to classify the strains used in our experiments, using serological methods.

In contrast to the definite pattern of growth curve we have found and which other (5, 6) workers have reported, Hill & Thornton (7) report highly erratic fluctuations in counts on five cheeses examined at seven stages up to 44 weeks. In no case was a consistent pattern evident, while in one cheese (no. 4) counts fluctuated back and forth between zero and over 100,000/g. The size of sample examined may offer at least a partial explanation of their results.

That the distribution of bacteria in cheese is far from homogeneous has long been recognized. In view of this it seems obvious that the sampling error should be minimized by examining as large a portion of the sample as can conveniently be handled. Mabbitt & Zielinska (5) used a 9 g. sample; Naylor & Sharpe (6) examined 8–9 g. from each of three cores; we customarily triturated approximately 10 g. and took a 5 g. aliquot for plating; Hill & Thornton examined only 1 g. portions. If, as it appears, their main interest was to determine *trends* in counts of lactobacilli during ripening, surely larger samples would have reduced variability and given less misleading results.

SUMMARY

The numbers of lactobacilli present in milk for cheese-making and in the cheese at various stages of ripening, have been determined for 38 Cheddar cheeses made during studies on flavour enhancement. These organisms multiplied rapidly even during the first few days of curing. Maximum levels were attained at 3–6 months; at 1 year counts had declined appreciably.

Flavour intensity in the experimental cheese appeared to be correlated with the level of lactobacilli present in (*a*) milk at the start of cheese-making and (*b*) at subsequent stages of ripening. These two count levels were usually closely correlated. Factory raw milk had the highest counts and gave the highest degree of flavour, followed by similar milk pasteurized and inoculated with selected strains of lactobacilli.

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STUDIES OF THE SECRETION OF MILK OF LOW FAT CONTENT BY COWS ON DIETS LOW IN HAY AND HIGH IN CONCENTRATES

VII. THE EFFECT OF ADMINISTRATION OF VOLATILE FATTY ACIDS TO COWS GIVING NORMAL MILK AND MILK OF LOW FAT CONTENT

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(With 6 Figures)

In the previous experiment of this series it was shown that with diets low in hay and high in concentrates the weight of acetate in the reticulo-rumen of the cow was lower, and the fluctuation in its concentration was greater, than in control periods with normal amounts of hay. It seemed possible, therefore, that the amount of acetic acid absorbed from the reticulo-rumen was small with diets low in hay and high in concentrates and that, as acetate is utilized as a source of energy and of milk fat by the mammary gland, the fall in milk fat percentage resulting from these diets might be due to a reduction in the amount of acetate available to the gland (1).

Workers at Wisconsin have briefly reported having, by the administration of acetic acid (2) or sodium acetate (3), but not of propionic acid (2), restored milk fat levels which had been depressed by diets low in hay. The present paper describes the results of five experiments carried out to determine the effect on milk composition of the administration of volatile fatty acids (mainly as sodium acetate) to cows receiving normal diets and diets causing low milk fat percentages. The results are also included of a sixth experiment in which cows received diets containing silage in place of hay, or fodder beet accompanied by a small amount of hay. Silage contains volatile fatty acids, and the sugars of fodder beet are likely to be rapidly fermented to these acids in the rumen.

METHOD

Five of the experiments (nos. 1, 2, 4, 5 and 6) consisted of experimental periods preceded and followed by control periods. Outlines of these five experiments and of Exp. 3 are given below and certain details are summarized in Table 1. The cows were from the Institute herd and were mainly Shorthorns. The additions of sodium acetate (CH_3COONa, $3H_2O$), sodium propionate (C_2H_5COONa) and sodium butyrate (C_3H_7COONa) were usually given mixed with the concentrates, and provided the additions were introduced gradually the cows ate them. In fistulated cows the additions were made by dripping solutions of these substances direct into the rumen. When changing to diets low in hay the change was gradual and was spread over 2 or 3 weeks, but changes back to normal levels were abrupt.

Exp. 1. *The effect of* (*i*) *adding sodium acetate to a normal diet and* (*ii*) *substituting molasses for flaked maize in a diet low in hay.* Twelve cows, which included three Friesians and one

Table 1. *Outline of treatments in Exps.* 1, 2, 4 *and* 6

Exp.	No. of cows	Period I (control) Weeks	Group	Diet	Period II (experimental) Weeks	Group	Diet	Period III (control) Weeks	Group	Diet
1	12	1–2	A	12 lb. hay, 30 lb. kale, mix. X*	3–7	A	As in period I	8–10	A	As in period I
		1–2	B	12 lb. hay, 30 lb. kale, mix. X*	3–6†	B	As in period I with 500 g. acetate	7–10	B	As in period I
		1–2	C	12 lb. hay, 30 lb. kale, mix. X*	3–7†	C	4 lb. hay, 10 lb. molasses, mix. P*, 8 lb. mix. Q	8–10	C	As in period I
2	2	1–3	—	18 lb. hay, 20 lb. mix. X	4–14	—	6 lb. hay, 20 lb. mix. X, 500 g. acetate (2 weeks)	15–18	—	18 lb. hay, 8 lb. mix. X
4	6	1–5	A	16 lb. hay, mix. X*	6–18	A	3 lb. hay, mix X*, 8 lb. mix. Y	19–21	A	16 lb. hay, mix. X*
		1–5	B	16 lb. hay, mix. X*	6–18	B	3 lb. hay, mix. X*, 8 lb. mix. Y	19–21	B	16 lb. hay, mix. X*
							Additions to diet:			
					12–14	A	558 g. acetate			
					12–14	B	414 g. propionate			
					15–16	A	414 g. propionate			
					15, 16	B	558 g. acetate			
6	18	1–3	A	16 lb. hay, mix. Z*	4–10	A	As in period I	11–13	A	As in period I
		1–3	B	16 lb. hay, mix. Z*	4–10	B	50 lb. silage, mix. X*	11–13	B	As in period I
		1–3	C	16 lb. hay, mix. Z*	4–10	C	60 lb. fodder beet, 6 lb. hay, 2 lb. groundnut cake, mix. Z*	11–13	C	As in period I

* Concentrates fed liberally according to yield, usually 4·0–4·5 lb./10 lb. milk produced.
† In group B week 3, and in group C weeks 3 and 4, were devoted to gradual changes in diet.

Guernsey, were blocked in threes according to milk fat percentage and stage of lactation in the initial control period, and were then allocated at random to the three treatment groups as indicated in Table 1. The molasses was introduced very gradually over 2 weeks into the diet of group C. Thus this group received a diet low in hay and high in soluble carbohydrate but containing no maize.

Exp. 2. *The effect of adding sodium acetate to a diet low in hay.* The necessary details are given in Table 1.

Exp. 3. *The effect of adding sodium butyrate to a diet low in hay.* One cow received a diet of 5 lb. hay and 17 lb. concentrate mixture X for 3 weeks before the experiment, and for the first 48 days of it. When the milk fat content had fallen she was given 300–500 g. sodium butyrate daily for two periods of 7 days. In a final control period she received 18 lb. hay and 17 lb. concentrate mixture X daily.

Exp. 4. *The effect of adding sodium acetate or sodium propionate to a diet low in hay.* Details are given in Table 1. The cows were paired according to the fall in milk fat percentage by week 11 and allocated at random to treatment groups A and B. Gradual additions of acetate or propionate were made in week 12. A liberal allowance of concentrates was given in period II in an attempt to maintain milk yields.

Exp. 5. *The effect of adding sodium acetate and other supplements to a diet low in hay.* Two fistulated cows (D 15 and K) were placed on experiment 3 weeks after calving. In the main experiment, which occupied 121 days, there was no control period, reduction of the amount of hay in the ration having begun even before calving. By day 22 both cows were receiving 2 lb. hay and 22 lb. proprietary dairy cubes. As this had been effective in reducing the milk fat percentage only in cow D 15, both cows were given an additional 8 lb. flaked maize. This was the basic diet for the period of experimental treatment extending from day 22 to day 91; it was replaced by 16 lb. hay and 22 lb. cubes in a final control period which continued until day 121. During the period of experimental treatment solutions containing 1 kg. sodium acetate daily were dripped into the reticulo-rumen of cow D 15 for 17 days and the amount was then increased to 1·5 kg. for 15 days. Cow K received 1 kg. sodium acetate for 18 days.

After the main experiment cow K was removed and the diet of cow D 15 was changed to 2 lb. hay, 20 lb. dairy cubes and 8 lb. flaked maize for 55 days, during which the milk fat percentage fell and various additions were made to the rumen. These additions were: (i) 500 g. acetate and 110 ml. acetic acid, (ii) 200 g. acetate, 300 g. propionate and 110 ml. acetic acid, (iii) 300 g. butyrate, 200 g. acetate and 110 ml. acetic acid, and (iv) double amounts of the additions in (ii). The cow then received 16 lb. hay and 8 lb. concentrates for 24 days.

Exp. 6. *The effect of diets containing silage and no hay, or fodder beet and little hay.* The eighteen cows (Table 1) included five Friesians and two Guernseys. They were blocked in trios according to date of calving and then allocated at random to the three treatment groups. Blocks were placed on experiment as they became available, this being usually between 2 and 4 weeks after calving. Each block followed the programme outlined in Table 1.

Foods. The hays used in these experiments were composed of ley grasses and clovers and were usually of average quality. The constituents of the various concentrate mixtures are given in Table 2. The dairy cubes used in Exp. 5 were of a proprietary brand.

The management of the cows and the sampling and analytical procedures were as in our earlier experiments (1, 4).

Table. 2. *The constituents of the concentrates* (%)

Ingredient	Mixture X	Z	P	Q	Y
Flaked maize	50	—	—	—	30
Wheat feed	35	—	88	—	20
Decorticated groundnut cake	15	10	—	—	—
Bran	—	20	—	—	—
Dredge corn (oats and barley)	—	60	—	—	—
Cottonseed meal	—	10	12	38	—
Beet pulp	—	—	—	62	10
Barley meal	—	—	—	—	30
Dried brewer's grains	—	—	—	—	10

2% Minerals were added to each mixture.

RESULTS

Exp. 1. There was no statistical difference between the milk fat percentages with any of the treatments, suggesting that neither the addition of acetate to a normal diet nor the inclusion of molasses in a diet low in hay had any effect. There was also no effect on the yield of milk or milk fat or on the solids-not-fat percentage.

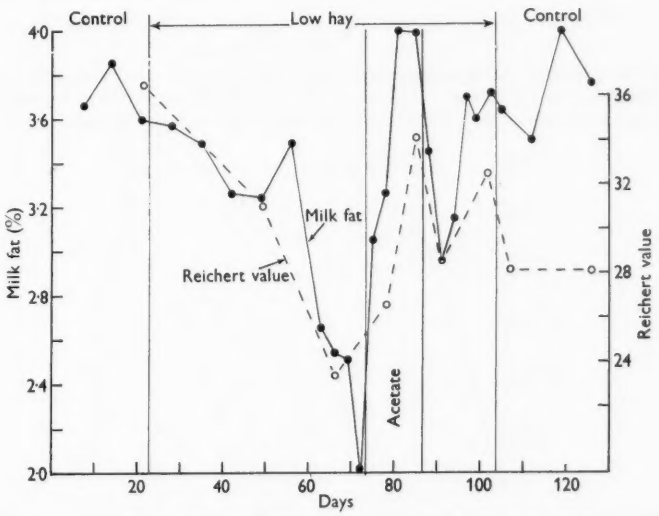

Fig. 1. Exp. 2. The fat content, ●—●, and Reichert value of butterfat, ○- - -○, of milk from cow D 15 receiving control diets with normal amounts of hay and a low hay diet of 6 lb. ley and 20 lb. concentrate mixture X. From days 72–85 the low hay diet was supplemented with 500 g. sodium acetate daily.

Exp. 2. Values for fat percentage are given in Figs. 1 and 2. With cow D 15 (Fig. 1) there was a steady fall in fat percentage and in Reichert value with the diet low in hay, but this trend was reversed by the addition of acetate to the diet. On the cessation of acetate administration both fat percentage and Reichert value fell, but there was an unexplained recovery in the last few days before the final control period. By comparison with the preliminary and final control periods, acetate administration appears to have brought about a complete recovery in both fat percentage and Reichert value.

With cow L 26 (Fig. 2) the results were obscured by the very advanced stage of lactation. Although giving 30 lb. milk daily at the beginning of the experiment her yield had declined to 16 lb. at the start of acetate administration and to 5 lb. by the end of the

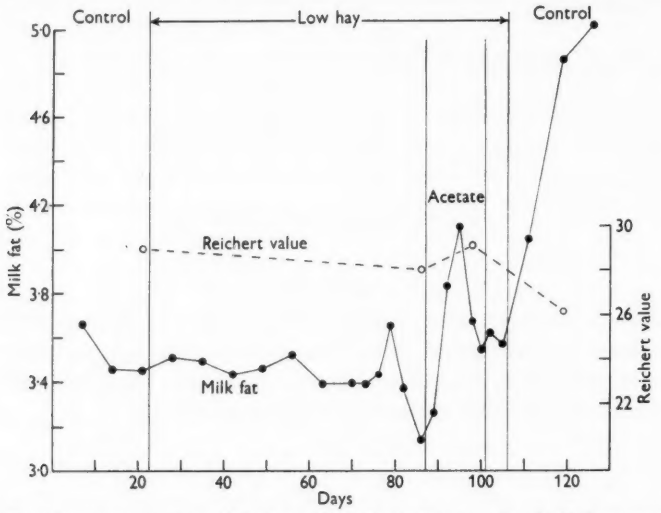

Fig. 2. Exp. 2. The fat content, ●—●, and Reichert value of butterfat, ○- - -○, of milk from cow L 26 receiving control diets with normal amounts of hay and a low hay diet of 6 lb. hay and 20 lb. concentrate mixture X. From days 87 to 101 the low hay diet was supplemented with 500 g. sodium acetate daily.

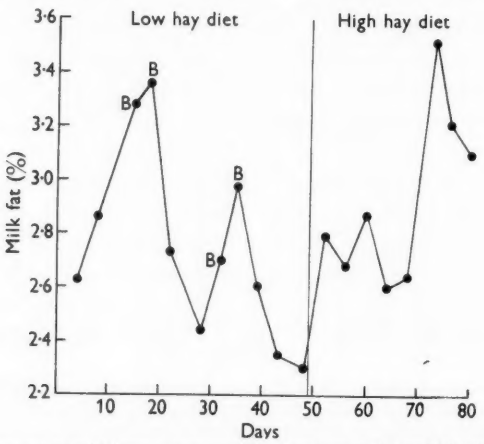

Fig. 3. Exp. 3. The fat content of milk from a cow receiving low hay (5 lb.) and high hay (18 lb.) diets. During the periods for which mean values are marked (B) the low hay diet was supplemented with 300–500 g. sodium butyrate daily.

experiment. Although there appeared to be no decline in fat percentage with the diet low in hay this may have prevented the usual late lactation rise, and there appeared to be a recovery of about 0·5% when acetate was given.

Exp. 3. The observations given in Fig. 3 suggest that in a cow in which the fat percentage was depressed by a diet low in hay and high in concentrates a recovery of 0·5–0·6% was

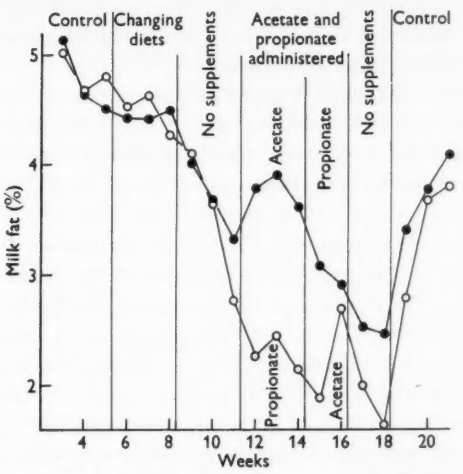

Fig. 4. Exp. 4. The mean fat content of milk from cows in groups A, ●, and B, ○, receiving control diets containing 16 lb. hay and concentrates or an experimental diet containing only 4 lb. hay with additional concentrates. Periods of acetate (558 g. daily) and propionate (414 g. daily) administration are indicated.

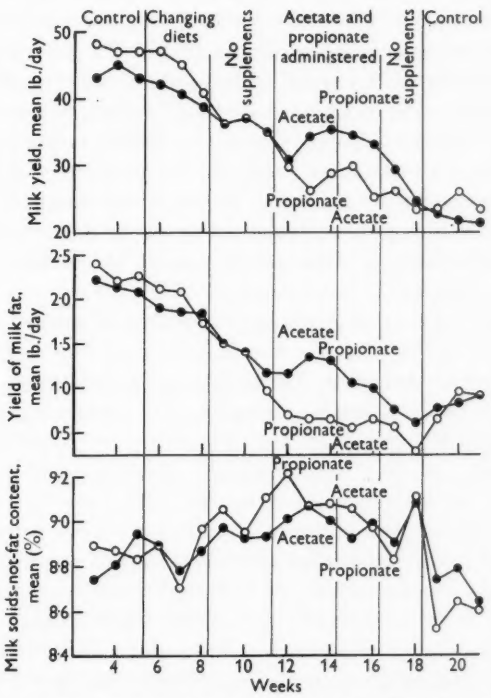

Fig. 5. Exp. 4. Mean values for the milk yield, yield of milk fat and the milk solids-not-fat content in groups A, ●, and B, ○, receiving control diets containing 16 lb. hay and concentrates or an experimental diet containing only 4 lb. hay with additional concentrates. Periods of acetate (558 g. daily) and propionate (414 g. daily) administration are indicated.

brought about by the administration of 300–500 g. sodium butyrate daily. Since with normal diets this cow produced milk of 3·1–3·5% fat, this recovery appears to have been complete in the first period of administration.

Exp. 4. In assessing the results it appeared that in one of the cows in group B the introduction of the diet low in hay did not cause a fall in fat percentage, probably owing to a low milk yield. Results for this cow have accordingly been discarded and the mean values shown in Figs. 4 and 5 are for three cows in group A and two in group B. The slow decline in the initial control period was probably the usual effect of early lactation and probably persisted into the change-over period. A remarkable fall in fat percentage was observed with the diet low in hay, values falling in group A from 4·8 to 3·4% and in group B from 4·8 to 2·8% by week 11. In both groups the administration of acetate appears to have reversed this decline, the reversal being apparent in values for four of the five cows. In none of the cows could such an effect be detected during the administration of propionate. In neither group did 558 g. acetate appear to bring about a complete recovery in fat percentage, although in group A the recovery may have been within 0·3%. While propionate was being administered and during weeks 17 and 18, when there were no supplements, the decline continued, reaching 2·4% fat for group A and 1·6% for group B.

The mean daily yields of milk from cows in groups A and B are shown in Fig. 5. There was a distinct tendency for milk yields of cows in group A to improve during the period of acetate administration, and also in the period of propionate administration the rate of decline was less marked than in weeks 17 and 18, but the response was less marked in group B. These values for yield, coupled with the effect on fat percentage, caused a considerable reversal of the usual declining gradient in fat yield, during the period of acetate administration. This was not apparent during the administration of propionate.

Also shown in Fig. 5 are the mean values for the percentage of solids-not-fat. There was an irregular increase throughout the period of experimental treatment, but the administration of acetate or propionate did not appear to influence these values. In our earlier experiments, increases in solids-not-fat content have usually been observed with diets causing severe depressions in fat content (1, 4, 5).

Exp. 5. With cow D15 the diet low in hay caused the fat content to fall to a relatively constant value of 1·7–2·0% (Fig. 6). Administration of 1 kg. sodium acetate daily in solution had no effect on this value. The acetate was administered, for various periods: (i) into the rumen by constant drip beginning 4 hr. after eating began; (ii) into the rumen in two equal amounts during feeding; and (iii) into the reticulum during feeding. None of these methods had any effect. Similarly when, after the end of the main experimental period, a diet low in hay was again given, the fat content averaged about 1·8–2·0% and was not changed in spite of the addition of various mixtures of volatile fatty acids. Double amounts of addition (ii) caused distress to the cow and she was found breathing heavily with condensed water vapour on her coat as though through sweating.

With cow K (Fig. 6) the addition of 1 kg. sodium acetate caused a substantial recovery of over 1·0% in fat content, irrespective of the method of administration. This was to within 0·3% of the level in the control periods. There was, therefore, a great difference between the responses of cows D15 and K.

The response of the Reichert value to the addition of acetate was similar to that of the fat percentage, there being no response shown by cow D15 whereas cow K showed what

appeared to be, when the lactational decline was considered, a complete recovery during acetate administration.

The values for solids-not-fat content in Exp. 5 are also given in Fig. 6, and for cow D 15 they show a marked increase when the milk fat percentage was depressed. Although this cow was late in lactation and barren, the low level (7·4 %) reached at the end of the experiment was exceptional. In cow K the solids-not-fat percentage varied little throughout the experiment.

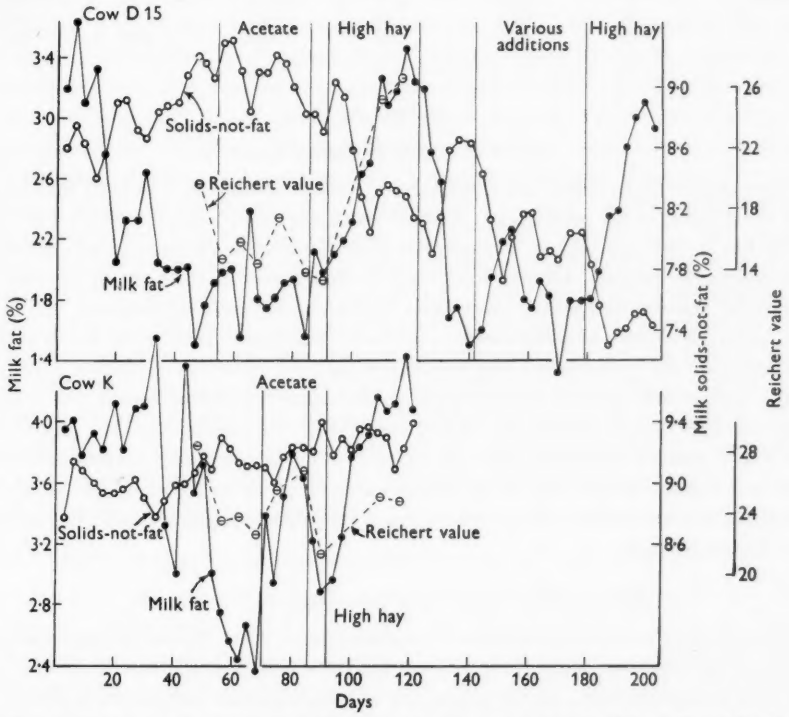

Fig. 6. Exp. 5. The fat percentage, ●—●, and solids-not-fat percentage, ○—○, of the milk and the Reichert value of the milk fat, ⊖----⊖, in cows D 15 and K receiving diets low in hay and high in concentrates except where indicated (high hay). Details of the amounts of acetate given as a supplement to the low hay diet between days 54 and 85 and of the various additions between days 141 and 178 are listed on p. 164. Each value for milk fat and solids-not-fat percentage is the mean for a period of 3 or occasionally 4 days.

Exp. 6. Mean values for the yield and composition of the milk produced during the preliminary control period (weeks 2 and 3) and the experimental periods (weeks 6–10) are given in Table 3. The statistical analysis included a direct comparison of treatment effects as shown in Table 3 and comparison of the mean value over experimental weeks 6–10 adjusted for initial difference by use of the mean value in weeks 2 and 3 as the auxiliary variable in the analysis of covariance. In neither instance were any of the differences between treatment group A and treatment groups B and C significant at the 10 % level.

Table 3. *The mean values in Experiment 6 for milk yield and composition during the preliminary control period (weeks 2 and 3) and experimental periods (weeks 6–10)*

Weeks of experiment	Treatment group	Daily yield, lb.			Milk composition (%)	
		Milk	Milk fat	Milk solids-not-fat	Fat	Solids-not-fat
2 and 3	A	42·9	1·76	3·69	4·07	8·62
	B	38·0	1·49	3·34	3·91	8·82
	C	39·9	1·67	3·49	4·25	8·83
6–10	A	37·6	1·53	3·21	4·06	8·56
	B	36·3	1·42	3·22	3·86	8·89
	C	34·2	1·37	3·02	4·09	8·93

DISCUSSION

Effect of acetate administration

The experiments have shown that addition of sodium acetate to a normal diet brought about no increase in fat percentage. Neither was there an increase if acetate were added to diets low in hay and high in concentrates and which for some reason had not brought about falls in fat content. Of nine cows treated while receiving diets low in hay and after the milk fat content had fallen, seven cows showed an appreciable response, but in only two of them did acetate administration raise the fat percentage fully to the levels obtained in control periods. This finding suggests either that the amounts of acetate given (0·5–1·5 kg.) were insufficient for this purpose or that some additional factor was involved. Possibly linked to this discrepancy is the observation that when cow D15 (Exp. 5) was given a diet which lowered her milk fat content to under 2·0% and maintained it there for several weeks, the addition of acetate in amounts of up to 1·5 kg. daily, or of varied mixtures of acetate, acetic acid, propionate and butyrate brought about no improvement in the fat percentage.

Effect of administration of propionate and of butyrate

Of the six cows to which propionate was administered none showed an improvement in fat percentage although one of these was cow D 15 in which there had also been no response to acetate administration. In the single cow to which sodium butyrate was given alone, the response was of the order often observed with acetate. Butyrate was also included in the mixtures, with acetate and propionate, which were administered without result to cow D15.

Comparison with earlier experiments

Although the responses to the administration of acetate were by no means as regular as might be desired in either their magnitude or their persistence, these experiments provide confirmation for the theory advanced previously (1) that diets low in hay and high in concentrates cause lowering of fat percentages by changing the relative proportions and probably the amounts of the volatile fatty acids produced by fermentation in the reticulo-rumen. It was postulated that since in ruminants acetate is absorbed from the reticulo-rumen and is known to be a source of a part of the milk fat (6), being built into saturated fatty acids containing up to 16 carbon atoms (7), a lowered production of acetate in the reticulo-rumen of cows receiving diets low in roughage and high in con-

centrates or a wasteful oxidation of acetate by such cows (1) could be the cause of the fall in fat content found with these diets. If a deficiency of acetic acid were the cause of the depressions in fat percentage it should be corrected by the administration of sodium acetate. In the present experiments the addition to the diet of 500 g. or more of sodium acetate was found to cause a considerable though not complete recovery in milk fat percentage. It was not possible to determine whether this amount was sufficient to restore rumen acetate levels to those found with the control diets. In experiments at Wisconsin 454 g. (1 lb.) of sodium acetate were found to give recovery in fat percentage (3), but until it is known whether that recovery was complete and whether the cows were giving more or less milk than ours, quantitative comparison of the results of the two experiments is impossible. As the peak of acetate production by rumen micro-organisms occurs some 2–4 hr. after feeding, it is probable, as was discussed earlier (1), that maximum utilization of added acetate would be obtained if it were given when fermentation in the reticulo-rumen was declining, although this was not effective with cow D 15 in Exp. 5.

By comparison with normal ratios of acetate to propionate in the reticulo-rumen the amount of added propionate was proportionally at least twice as great as the amount of added acetate, yet the experiment demonstrated that the acetate had, and propionate had not, the ability to improve the low fat percentages. However, apart from milk fat synthesis both acetate and propionate are well known to be important sources of energy in the body. It is, therefore, extremely interesting to note that the administration of acetate sometimes caused an improvement in milk yield and that, in Exp. 4, this was partly maintained in succeeding periods of propionate administration. At these times the diets were by normal standards ample for the amount of milk being produced.

In our earlier experiments (1, 4, 5), increases in solids-not-fat content have usually been observed with diets causing severe depressions in fat content. The same tendency was observed in the present experiments.

Inclusion of silage and fodder beet in experimental diets (*Experiment* 6)

In none of the six cows receiving silage in Exp. 6 was there a fall in milk fat content. The silage supplied about 13 lb. dry matter, but was very succulent. We have often been asked about the possibility of low fat percentages with similar diets, and farmers and others clearly suspect that these occur, but this was not found under our present experimental conditions. Where such low values are encountered we suspect they are due more to the succulence of the silage and consequent lack of 'fibrousness' in the whole diet than to any special property of the volatile fatty acids produced during ensiling. Zelter (8) gave cows a diet of 45 kg. fodder beet, 4 kg. hay and concentrates and then replaced the fodder beet with an equal weight of ensiled sugar beet tops. Following the change, increases were noted in both the yield and fat percentage of the milk. This amount of silage contained 480 g. acetic acid and 243 g. butyric acid. In further experiments replacement of fodder beet by silage and supplementation of the diet containing fodder beet with calcium acetate or butyrate, in the amounts found in the second silage, either alone, mixed together or in mixture with pyruvate, exerted no effect on fat percentage. In our Exp. 6 it was somewhat surprising that the change from a normal diet to one including 60 lb. fodder beet and only 6 lb. hay did not affect the fat percentage, because a lowered production of acetate in the rumen might have been expected with this diet.

SUMMARY

1. The administration of 0·5–1·5 kg. sodium acetate to cows in which the milk fat percentage had been reduced by diets low in hay and high in concentrates usually brought about an appreciable improvement in fat percentage. The extent of the response varied from slight to complete. The Reichert value of the milk fat fell with diets low in hay, but was raised by administration of acetate.

2. The daily administration of 500 g. sodium acetate to cows receiving diets containing normal levels of hay and concentrates according to their milk yields did not affect the milk fat content.

3. The daily administration of 414 g. sodium propionate did not restore fat percentages lowered by the diets low in hay. Butyrate appeared, in a test with one cow, to possess the restorative properties of acetate.

4. With diets containing either 50 lb. silage as the sole roughage or 60 lb. fodder beet and only 6 lb. hay, fat percentages were the same as with a normal diet containing 16 lb. hay.

We acknowledge with pleasure the help of Dr D. A. Balch in the planning of the earlier experiments and in the preparation of the paper, and of Miss Z. D. Hosking who carried out the statistical analysis of Exp. 1. We also thank Mr V. W. Johnson for his care of the cows and Mrs R. Winder and Mr A. W. Wagstaff for analysing the milk.

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THE EFFECT ON MILK COMPOSITION OF FEEDING SPRING GRASS TO COWS

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(With 1 Figure)

Many studies of the chemical composition of milk have shown seasonal variations which often appear to be associated with some change in the feeding of the cow(1,2). In the temperate climate of the British Isles the most abrupt seasonal change in milk composition is that which occurs when cows go from indoor winter feeding to graze spring grass. Within a few days the solids-not-fat (S.N.F.) content of the milk usually increases, often by as much as 0·3–0·4%, an increase which has been shown by several workers to result almost entirely from a rise in the protein content of the milk. There is also frequently a decline in fat percentage about the same time or a little later(1,2).

Since Riddet, Campbell, McDowall & Cox(3) and Rowland(4) showed that underfeeding reduced the S.N.F. content of a cow's milk, it has been suggested that herds may be underfed to some extent towards the end of the winter and that the change to a plentiful supply of high quality spring grass produces the change in S.N.F. content that might be expected from an increased plane of nutrition. Another explanation, current some years ago, was that the oestrogen content of spring grass caused hormone-induced changes in the cow's metabolism which gave rise to an increase in the S.N.F. content of the milk(5). However, oestrogens are either absent altogether or present in such negligible quantities in most pasture grasses(6), even in spring, that this hypothesis is now little favoured.

The ability of additional carbohydrate in the ration to bring about an increase in the S.N.F. content of the milk(7) suggested that one factor contributing to the effect of spring grazing on milk composition might be the soluble carbohydrate content of the grass. Several of the most widely used pasture grasses have been shown to contain more of these compounds, particularly of the polymer fructosan, in spring than at any subsequent time of the year(8). Direct additions of sucrose(9) and of starch((10) to a roughage diet fed to sheep and steers have been shown to depress fibre digestibility, particularly if the roughage was of low nutritional value. The position may, however, be different with fresh grass where adequate supplies of protein and other nitrogenous compounds will accompany the soluble carbohydrates. Such carbohydrates would be readily available to rumen bacteria and in addition to acting as a source of energy would rapidly provide molecular structures for precursors of other metabolites. This view received some support from the recent work of Lewis & McDonald(11), who showed that grass fructosan, fed at the same time as a source of protein, produced a very efficient type of fermentation in the rumen.

An experiment has been made, therefore, to determine whether spring grass fed indoors at the same plane of nutrition as the winter feed had any intrinsic effect on milk

composition. To avoid any chance of inadequate feeding whilst on winter rations, a level about 115% of the Woodman standard was adopted. It is realized that grass cut and fed to cows differs in chemical composition, and probably nutritive value, from grass that the cow would graze, but its use was preferred for a number of reasons. The chief of these was that the food intake can be estimated much more accurately, and supplemented when necessary. Moreover, the soluble carbohydrate content, particularly the fructosan fraction, would almost certainly be higher in cut grass than in the more leafy grass that cows select when grazing (8), and if this carbohydrate fraction is a major factor in raising the S.N.F. content in the spring the response should be more readily observed. At present it is not possible to say whether the increased length and intensity of daylight in spring affects the cow's metabolism in such a way as to alter milk composition, but exposure to direct sunlight and the exercise associated with grazing can be avoided by indoor feeding.

EXPERIMENTAL

The experiment was designed to compare the composition of the milk from two groups of cows. Group 1 (6 cows) was fed a good quality winter ration throughout the experiment, whilst group 2 (7 cows) had a 6-week period of grass feeding interposed between two periods of winter feeding. The aim was to keep the plane of nutrition of the cows of both groups always at the same level.

Animals. In late December 1957, 13 Ayrshire cows, due to calve in February 1958, were bought from a number of commercial herds in south-west Scotland. Their ages ranged from 4 to 10 years and averaged 6½ years. They were weighed once per week throughout the experiment.

Feeding. Before calving, all cows were given a daily maintenance ration of 9 lb. hay, 9 lb. dried beet pulp and 2 lb. oat straw and were steamed-up with a concentrate which contained 21% digestible crude protein (D.C.P.) and had a starch equivalent (S.E.) of 69 as fed. The concentrate was fed at the rate of 2 lb. per cow per day in the first week after purchase and increased by 2 lb./week until 10 lb. per cow per day were being fed. The 13 cows calved over a period of 6 weeks and, since steaming-up started for all cows on the same date, some of them received considerably more concentrates than others. This was unavoidable since the calving dates of the bought cows were known only very approximately. No cow received less than 130 lb. concentrates and the average weight fed prior to calving was 360 lb. After calving, the same maintenance ration was given plus 4 lb. concentrate/10 lb. milk. Food refusals of all feeds were weighed on 3 days/week.

When grass became available for feeding, the yield and composition of the milk of the 13 cows had been known for periods varying from 5 to 11 weeks. The cows were then divided into two groups, based mainly on the yield and S.N.F. content of their milk, with age and weight as secondary factors. Two groups with similar characteristics were obtained.

During the 6-week grass-feeding period for cows in group 2, the grass was cut three times per day, at 6.30 a.m., 11.30 a.m. and 4 p.m. One quarter of the total amount fed per day was given at 7 a.m., noon, 4.30 p.m. and at 7 p.m. Thus only the grass of the last feed had been cut for more than ½–1 hr. before it was eaten. To provide leafy grass likely to contain appreciable amounts of soluble carbohydrates, pure ryegrass or mixtures containing ryegrass were used.

The change from winter rations to the grass ration was made over a period of a week, at the end of which time 160–180 lb. fresh grass per day per cow were being offered. The grass left uneaten or wasted by each cow was removed before the next feed and weighed.

It was initially assumed, and early confirmed, that the cows would eat about 28 lb. grass dry matter per day and it was realized that this would not provide sufficient nutrients for the high-yielding cows. Since it was essential to maintain a constant level of feeding, when the estimated daily nutrient intake from grass was insufficient, concentrates were fed in addition. This resulted in 2 of the 7 cows receiving 9 lb. concentrates/day and 5 cows receiving 3½ lb. concentrates/day during the grass feeding period, an average for the group of 5 lb./day.

In the third experimental period the cows of group 2 returned, over a period of 4 days, to rations of hay, beet pulp and concentrates fed at the same rate as in period 1.

Spring grass was available on 25 April 1958, and the cut grass was estimated as being capable of supplying most of the food requirements of the cows in group 2 during the next 6 weeks. The results have therefore been calculated for the three experimental periods as follows:

	Period		
Cow group	1 (6 weeks)	2 (6 weeks)	3 (3 weeks)
1	Winter feeding	Winter feeding	Winter feeding
2	Winter feeding	Grass indoors	Winter feeding

Nutrient values of the feeds

The analyses and calculated nutrient values of the hay, beet pulp and concentrates are given in Table 1. Daily samples of each feed were taken and the protein content of the weekly composite sample determined. The hay and dried beet pulp were bought in single lots and the concentrates in three batches. Weekly analyses of all three feeds varied so little that a final composite sample, representative of each feed for the whole experiment, was made and analysed in the detail shown in Table. 1.

Table 1. *Analyses and approximate feeding values of the winter rations and the spring grass*

		% dry matter								
Feed	Dry matter (% feed)	Crude protein	Ether soluble	Crude fibre	Ash	Lignin	Total soluble carbo-hydrates	Fructo-san	D.C.P.*	S.E.*
Concentrate	87·1	24·2	5·8	8·7	7·9	4·8	N.D.*	N.D.*	20·6	78
Beet pulp	87·2	11·4	1·4	16·2	8·0	2·9	23·4	0	6·8	68
Hay	82·6	5·6	2·9	32·3	6·3	11·7	8·5	2·7	2·2	30
Oat straw	85·0	3·8	1·1	41·3	6·5	17·1	0	0	0·9	20
Grass 1	19·3	21·4	6·2	14·9	10·3	3·0	17·9	6·8	16·7	68
2	18·7	18·3	6·8	17·3	9·2	4·2	18·3	7·3	13·1	67
3	16·9	17·2	6·2	16·9	10·0	4·4	15·8	4·6	12·2	64
4	19·8	21·5	6·3	19·1	9·1	3·9	17·2	3·3	16·7	68
5	18·6	13·3	5·3	19·4	7·9	4·1	20·8	8·5	8·9	59
6	17·4	18·3	5·9	19·8	10·4	3·7	14·7	3·7	13·2	62
7	20·6	10·8	4·3	20·1	7·8	4·7	23·0	12·3	7·0	58
8	18·0	18·1	6·2	19·0	9·6	3·6	15·6	5·6	13·1	63
9	17·4	16·1	6·2	21·3	9·3	3·8	13·2	5·4	11·3	62
10	19·5	11·9	4·9	21·1	7·3	4·6	18·7	11·7	7·9	58

* D.C.P. = digestible crude protein. S.E. = starch equivalent. N.D. = not determined.

The nutritive value of the hay was estimated from Moon's (12) recent studies of Scottish hays and those of the beet pulp and concentrate were calculated from Woodman's (13) tables, basing the value for the concentrate on the D.C.P. and S.E. contents of the individual constituents and adjusting for the moisture and protein contents found. The rations were intended to provide about 115% of the Woodman standard, i.e. 7 lb. S.E. and 0·7 lb. D.C.P. for maintenance (1000 lb. live weight) and 2·9 lb. S.E. and 0·7 lb. D.C.P. for production of 10 lb. milk.

The moisture, crude protein and fibre content of the grass fed to cows in group 2 were determined each day on the grass cut at 6·30 a.m. The D.C.P. content was calculated from Dijkstra's (14) equation for spring grass and the S.E. value from the fibre content according to Hallsworth (15). The approximate feeding value for each day's grass was known by midday and determined the amount of supplementary concentrates to be fed to each cow at the evening milking. When grass was to be cut from a new field, a sample was taken the day before the grass was required and an estimate of its feeding value made.

The analyses of the grasses from the ten fields used are shown in Table 1. The D.C.P. contents of the grasses fed were re-calculated at the end of the experiment by the method of Schneider, Lucas, Pavlech & Cipolloni(16) using the fuller analyses then available and agreed well with the values previously obtained. The S.E. values in Table 1 were estimated by Kellner's method using digestibility coefficients for the ether-soluble material, crude fibre and nitrogen-free extractives based on a number of sources (e.g. (13, 15, 16)). As the crude fibre contents of the fresh grasses were less than 4%, a deduction of 0·29 S.E. for each 1% crude fibre was made.

The fructosan contents were low but probably as high as could be expected in young leafy grass cut with a mower which left a stubble of about 2 in.

Milk yield and composition. The cows were milked twice daily, and the weight of milk recorded at each milking.

Samples for analysis were taken from each cow three times per week; the evening and morning milks were sampled separately and mixed in proportion to yield. The milk was analysed for total solids and fat (giving S.N.F. by difference), lactose, total nitrogen, casein, albumin, β-lactoglobulin and non-protein nitrogen (N.P.N.). The albumin and β-lactoglobulin fractions were determined by adapting the preparative method of Aschaffenburg & Drewry (17), to routine analysis. The lactalbumin filtrate was prepared by adding the sodium sulphate as a 25% solution at 40° C., and the β-lactoglobulin filtrate by allowing the albumin precipitate to coagulate at 30° C,, when it could be easily removed by filtration. The N.P.N. content was determined according to Rowland (18) and the other constituents as previously described (1).

As an indication of the health of the udder, the differential cell count (19) was made on all milk samples, and in addition, weekly fore-milk samples from each quarter were taken for bacteriological examination.

RESULTS

The chemical composition of milk is known to change rapidly, both quantitatively and qualitatively, in the first few days after calving, and for several weeks thereafter the percentage of fat and the nitrogenous constituents usually continue to fall, whilst the lactose content rises. To avoid most of these rapid changes the results given below have been taken only after the fifth week of lactation.

Nutrient intake. The average daily dry matter intakes during period 1 (winter feeding) were 34·4 lb./cow for group 1 and 33·7 lb./cow for group 2. In period 2 these amounts had fallen to 30·9 lb./cow and 32·3 lb./cow respectively. The cows in group 2 ate on average 28 lb. grass dry matter/day in the 5-week period following the change-over week, with the intake of individual cows varying from 25 to 29 lb./day, a variation associated mainly with the weight of the cows.

In period 3, cows of group 1 ate 29·4 lb. dry matter/day and those of group 2 ate 31·2 lb., the difference being entirely in the weights of concentrates eaten by the two groups, the weights differing because of the lower average yield of group 1.

Table 2. *Calculated nutrient intake utilized for the production of* 10 *lb. milk*

(*Maintenance allowance*: 0·76 lb. D.C.P., 7 lb. S.E.)

	Period					
	1		2		3	
Group	D.C.P. (lb.)	S.E. (lb.)	D.C.P. (lb.)	S.E. (lb.)	D.C.P. (lb.)	S.E. (lb.)
1	0·7	2·9	0·8	3·0	0·7	2·9
2	0·7	2·8	0·8	3·0	0·7	3·0

In Table 2 the calculated nutrient intake for the two groups of cows has been expressed as the amount of nutrients used to produce 10 lb. milk after deducting maintenance requirements.

It will be seen that the object of securing a level and equal supply of nutrients to the cows in both groups was substantially achieved, within the well recognized limitations of the method of calculation.

Live weight. No allowance has been made in Table 2 for changes in the weight of the cows. The average weight of the cows in group 1 did not change throughout the whole experiment, remaining steady at 1060 lb. Cows in group 2 lost weight during period 2, from an average group weight of 1030 to 1000 lb., but in period 3 their average weight had recovered to the same value as in period 1. The loss of weight in period 2 when grass was being fed indoors is likely to have been the result of changes in rumen fill rather than loss of body tissue.

Health of the animals. The general health of the animals was good. Some temporary minor ailments affected 2 cows in each group but did not seriously influence the group average values for yield or composition of the milk. One cow in group 1 suffered from clinical mastitis for a few days in the middle of period 2 and several cows were treated with antibiotics when an abnormal cell count or the presence of pathogenic bacteria in the milk warranted it.

Milk yield and composition. In Table 3 the mean yield and composition of the milk produced by each group is given for subperiods of 3 weeks duration, starting with the last 3 weeks of period 1. The average stage of lactation at the beginning of the first of these subperiods was 58 days for group 1 and 55 days for group 2, the stage of lactation when fat and S.N.F. contents would be expected to have, approximately, their lowest values (1).

Considering first the results for the control group (group 1), which was fed on winter rations throughout the experiment, it can be seen that as the experiment progressed the chemical composition of the milk followed a normal lactational trend, both fat and S.N.F. content rising. The small increase in the S.N.F. content in period 2 was the result of a

Table 3. *The mean yield and percentage composition of the milk averaged over 3-weekly subperiods*

Group	Period	Yield (lb. per cow per day)	Fat	S.N.F.	Lactose	Crude protein	Casein	Total albumins	β-lactoglobulin	N.P.N.
1	1 (last 3 wk.)	49·3	3·53	8·72	4·64	3·18	2·44	0·44	0·25	0·15
	2 (first 3 wk.)	43·6	3·72	8·76	4·58	3·27	2·49	0·46	0·25	0·16
	2 (second 3 wk.)	41·1	3·72	8·78	4·60	3·28	2·52	0·45	0·27	0·16
	3 (first 3 wk.)	38·7	3·82	8·81	4·55	3·38	2·57	0·49	0·26	0·16
2	1 (last 3 wk. winter feeding)	50·1	3·57	8·68	4·70	3·07	2·37	0·41	0·24	0·16
	2 (first 3 wk. grass feeding)	45·7	3·75	8·83	4·69	3·22	2·50	0·43	0·25	0·17
	2 (second 3 wk. grass feeding)	44·8	3·70	8·87	4·72	3·24	2·51	0·44	0·25	0·16
	3 (first 3 wk. winter feeding)	42·1	3·81	8·88	4·70	3·25	2·51	0·44	0·25	0·16

(All the protein and N.P.N. values are calculated as N × 6·38.)

fall of 0·04–0·06% in lactose content minimizing a rise of 0·09–0·10% in crude protein content. In period 3 similar changes continued at a slightly greater rate.

The composition of the milk of the cows fed cut grass (group 2) was very similar to that of the control group during the last 3 weeks of winter feeding (period 1), although slightly lower in protein content. During period 2, the S.N.F. content increased by 0·15% in the first 3 weeks of grass feeding, with a further slight increase to 0·19% in the second 3 weeks. These increases resulted entirely from a rise in protein content and were contributed to proportionately by all the nitrogen fractions that were measured. There was no change in milk composition when these cows returned to winter rations for the final 3 weeks of the experiment (period 3). The marked rise in milk yield which frequently occurs at the beginning of spring grazing was absent from the yields of the grass-fed cows. The fall in yield and the rise in the fat contents were similar for both groups during the whole experiment, although both changes proceeded at a slightly greater rate in the milk of cows in group 1.

Since the increase in S.N.F. content in spring is mainly the result of a marked increase in protein (1), the values for this constituent in the milk of cows of both groups for 30 days before and after the date at which grass feeding to group 2 started are shown in Fig. 1. For comparison, curves showing the change in protein content over the same period of each lactation, derived from earlier work with 814 cows (1), have been drawn below the experimental points (without reference to the ordinate values). In these reference curves the effect of season had been statistically eliminated.

The figure shows that for both groups, the changes in protein content were essentially those associated with advancing lactation, and that feeding cut grass at the same plane of nutrition as the previous winter feed had little specific effect. A statistical comparison of the change in mean protein content between the first and second periods, by pairs of cows of the two groups, confirmed this finding.

To determine whether the usual marked seasonal rise in the protein content of the milk of cows fed typical farm winter rations had, in fact, occurred during the course of the experiment, grass from the same fields as those used to provide the cut grass was grazed by cows of the Institute's herd. Analysis of the milk from a small group of these cows for

some weeks before and after the start of spring grazing showed that the protein content of their milk increased as soon as grazing provided the major portion of their diet, and that at the end of 14 days it was 0·4% higher than during the last 3 weeks indoors. It can therefore be assumed that the effect of spring grazing in 1958 on the milk composition of cows fed a normal winter ration of hay, silage and concentrates (calculated to provide slightly above the Woodman standard) was normal.

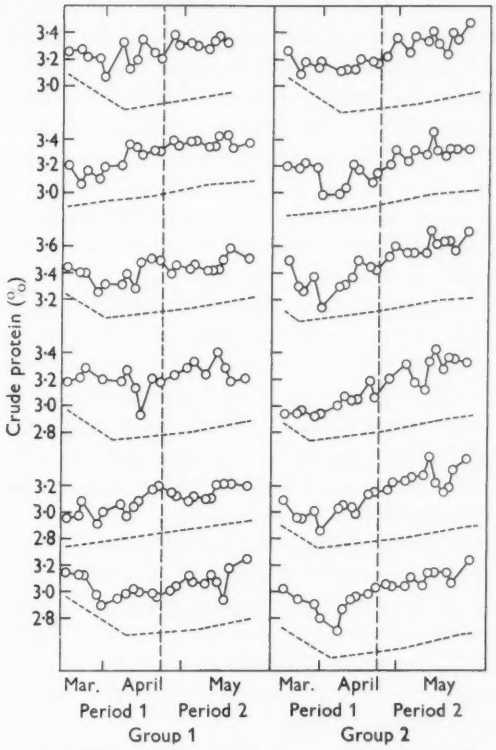

Fig. 1. The crude protein contents of the milk of individual cows in periods 1 and 2. (The dotted curves are taken from analyses of the milk of 814 cows, Waite *et al.* (1) and show the effect of stage of lactation only; see text).

Throughout the experiment the relative proportion of the various nitrogen fractions measured remained extremely steady, the average values and ranges (expressed as a percentage of the total N) during 15 weeks being:

Casein N	77·9 (77·6–78·4)
Total albumin N	13·6 (13·3–13·8)
β-Lactoglobulin N	5·8 (5·5– 6·1)
N.P.N.	5·0 (4·8– 5·4)

DISCUSSION

The marked rise in milk protein content which usually accompanies the start of spring grazing, and which was observed in the milk of the herd cows whilst the present experiment was in progress, did not occur when cut grass was fed indoors. The rise in the protein

content of the milk of the grass-fed cows, although small, was, however, greater than that observed in the milk of the cows fed continuously on winter rations (0·17% compared with 0·10%).

Since the quality of the milk of all the cows in their previous lactations was unknown, it would be wrong to place undue emphasis on this small difference, particularly as it depends for validity on calculations of nutrient intakes which cannot be other than very approximate. Nevertheless, recent work (10) has shown that addition of protein and a water-soluble polysaccharide such as fructosan to the rumen of sheep produced less free ammonia and a higher proportion of propionic acid than when starch was the added carbohydrate. The cows eating cut grass received about 5 lb. soluble sugars each per day, of which about 2 lb. was fructosan, and the increased fermentation efficiency induced by this diet may have contributed to the small difference in milk protein content of the two groups.

The failure to show, even temporarily, any appreciable rise in milk protein or S.N.F. contents when the ration of the group 2 cows changed from winter rations to cut grass, at what was substantially a constant nutrient intake, supports the view that when such a rise does occur it probably results from an improvement in the general plane of nutrition. Since an increase in milk protein content can be brought about most readily by increasing the energy content of a slightly deficient diet (3, 4), the seasonal change to spring grazing most likely results in an improved energy intake and utilization. The possibility cannot be overlooked, however, that when animals go to spring grazing after some 6 months indoors, there may be an improvement in their general condition, with a greater endocrine activity of the type which affects not only the yield of milk produced but also its composition. Further experimental work is required.

Soon after the start of spring grazing, milk yield frequently shows a marked temporary increase with a corresponding depression of fat content. Neither of these reactions was shown in the milk production of the grass-fed cows. Additional factors tending to prevent any lowering of the fat content may have been the absence of any decrease in the fibre content of the diet (5·0 lb./cow/day on winter feed and 5·7 lb./day when fed cut grass), and the continued supply of some concentrates which would prevent an abrupt change in the number and types of rumen bacteria.

SUMMARY

1. An experiment to investigate the seasonal increase in the solids-not-fat content of milk, which usually occurs in Britain when cows go from winter feeding to spring grazing, is described.

2. Two similar groups of cows were fed good quality winter rations at the same high level both before and after calving (about 115% of the Woodman standard after calving), until spring grass was available. One group of 7 cows was then fed for 6 weeks almost entirely on cut grass, returning to winter rations for a further 3 weeks. The control group of 6 cows continued to be fed throughout the experiment on winter rations and both groups remained indoors for the whole of the experiment. The estimated nutrient intake of both groups in all periods was kept substantially constant.

3. Changes in milk yield and composition were mainly those associated with advancing stage of lactation, and grass feeding had little specific effect. It is concluded that the

increase in milk protein content which is normally observed soon after the start of spring grazing probably results from an improved plane of nutrition and not specifically from an increase in the intake of soluble carbohydrates.

4. Increases in the total nitrogen content of the milk as the experiment proceeded were contributed to uniformly by the casein, total albumin and β-lactoglobulin fractions.

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RELATIONS BETWEEN COMPOSITION AND VISCOSITY OF COW'S MILK

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Theoretical and practical aspects of the apparent or effective viscosity of milk have been studied by many workers: see reviews by Scott Blair (1, 2, 3). Seidler & Elke (4), Cox (5), and others have considered the relations between observation temperature and viscosity measurements; Mohr & Oldenburg (6) and Puri & Gupta (7), for example, have investigated the effects of differences between individual viscometers. Other workers, including Puri & Gupta (7), Kobler (8) and Spöttel & Gneist (9), have examined the viscosity of whole raw milk after different times and temperatures of storage; and Weinlig (10), Trout & Weinstein (11) and Whitnah, Rutz & Fryer (12, 13) have studied changes in viscosity values in connexion with various processes such as pasteurization and homogenization.

The outcome of researches on the relationship between viscosity and composition appears, however, to be less definite although it seems now to be generally accepted that the protein, and particularly the casein content, is of great importance (Puri & Gupta (7), Kobler (8), Spöttel & Gneist (9)). One equation connecting viscosity (measured after the addition of ammonia) with the protein percentage of skim-milk, indicating an increase in effective viscosity with increased protein content, has been recently derived by Puri & Gupta (14) who considered that their expression would also apply to whole milk. Direct evidence, however, on the relationship between viscosity and protein percentage in whole milks seems to be rare.

The effect of the presence of fat on the viscosity of milk has been investigated by Mohr & Oldenburg (6, 15), Spöttel & Gneist (9) and Tapernoux & Vuillaume (16), for example, who found that the effective viscosity of whole milk was higher than that of skim-milk, and by Leviton & Leighton (17) who used emulsions containing cream and skim-milk and derived an empirical formula describing the increase in viscosity with the increase in fat concentration. Even if the relationship between viscosity and percentage of fat is of primary interest, it must be noted that the influence of fat may not depend simply on its concentration in the milk, and for more fundamental studies the size distribution of the fat globules will require consideration; for example, the work of Mohr & Oldenburg (15) suggests that fat particles of abnormally large size have no appreciable effect on the viscosity of milk whereas clusters of fat globules of irregular form increase viscosity.

The relationship between viscosity and total solids percentage has been studied by Eilers, Saal & Van der Waarden (18) using concentrated skim-milk, by Torssell, Sandberg & Thureson (19) using both skim-milk and standardized (3% fat) milk, and Seidler & Elke (4), Spöttel & Gneist (9) and Kooper (20) using whole raw milk. The resu ts generally

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indicate that effective viscosity at a given temperature increases with increasing total solids concentration.

An early empirical formula connecting viscosity with both the fat and solids-not-fat percentages was given by Taylor(21): this has been widely quoted in standard texts without an accompanying qualification that the formula was based on the results of a single sample only. Apart from this and the more recent researches of Kulkarni & Dole(22), no references to the relationship between viscosity and the separate composition factors have been found.

In view of the complex nature of milk, no theoretical treatment has been attempted in the present paper where attention has been confined to estimating empirical equations showing the regression of viscosity on the percentages of fat and of solids-not-fat. Further, because of the differences in the circumstances of reported experiments and particularly in the viscometers used, the regressions calculated from the data of different investigations are presented separately.

REVIEW OF PUBLISHED DATA

In this section, only summaries of the experimental methods and results are given and for more details the original publications should be consulted. To facilitate comparisons between different sets of published data, relative viscosities have been multiplied by the viscosities of water at the corresponding temperatures, that is converted to absolute viscosities, e.g. Davis(23). In addition, the following standard notation has been adopted throughout.

$\eta_{\text{rel.}}$ = relative viscosity;
η_m, η_w = absolute viscosity in centipoise of milk and water respectively;
ρ_m, ρ_w = density of milk and water respectively;
s_m, s_b = specific gravity of milk and viscometer ball respectively;
t_m, t_w = time of flow of milk and water respectively (capillary viscometer);
t_b = time of fall of ball (Höppler viscometer);
k = constant for particular conditions of an experiment.

Taylor(21)

An Ostwald-type capillary viscometer was used for determining the relative viscosity as given by the formula*

$$\eta_{\text{rel.}} = \frac{t_m \rho_m}{t_w \rho_w}$$

Viscosity determinations were made at 20° C. on samples of cow's milk within 4 hr. of milking. The total solids percentage of the milk samples was determined by drying in a steam oven for 3 hr.; the concentrations of fat and solids-not-fat were tabulated for individual samples.

A summary of the observed results and of the derived absolute viscosities is given in Table 1. It may be noticed from the composition values that all the milks examined had high fat percentages and the differences between milks were small both in fat and solids-not-fat content.

* In Cox(5), p. 75, the ρ components of this formula were accidentally inverted.

Table 1. *Viscosity and composition values obtained from* 12 *samples of cow's milk* (21)

	Mean	Range
Relative viscosity ($\eta_{rel.}$ at 20° C.)	1·999	1·95–2·06
Viscosity* (η_m cP. at 20° C.)	2·003	1·95–2·06
Fat (%)	5·26	4·70–5·75
Solids-not-fat (%)	9·32	9·10–9·60

* Estimated from formula $\eta_m = 1{\cdot}0019\ \eta_{rel.}$

Kooper (20)

A special design of capillary viscometer (see original text) was used. Viscosity constants, V, were calculated from the formula

$$V = \frac{t_m}{t_w}$$

and it can be seen from the standard expression for relative viscosity quoted in the preceding section, that the viscosity constant, also known as the relative kinematic viscosity, is equal to the relative viscosity divided by the specific gravity of the milk. Flow times were obtained under room temperature conditions within the range 17–20° C. so that although testing temperatures for individual samples were not reported, it appears reasonable, for present purposes, to accept the published values as measures of V at 18·5° C. Fat concentration in the milk samples was determined by the Gerber method and specific gravity by lactometer readings. The total solids percentages were then calculated by Fleischmann's formula.

For a few samples V was not identically reported in different tables and, where this occurred, those values in best agreement with others of Kooper's figures have been used. Experimentally determined viscosity constants have been converted to absolute viscosities and are shown, together with composition values, in Table 2.

Table 2. *Viscosity and composition values of cow's milk* (20)

	Individual milks		Bulk milks	
	Mean	Range	Mean	Range
Viscosity constant (V at 17–20° C.)	1·788	1·38–2·31	1·591	1·38–1·73
Relative viscosity* ($\eta_{rel.}$ at 17–20° C.)	1·841	1·42–2·37	1·638	1·42–1·78
Viscosity† (η_m cP. at *c.* 18·5° C.)	1·914	1·48–2·46	1·703	1·48–1·85
Fat (%)	4·53	2·30–7·50	3·09	2·20–3·95
Solids-not-fat (%)	8·56	7·82–9·74	8·39	7·72–8·98
No. samples	19		50	

* Estimated from formula $\eta_{rel.} = Vs_m\ (= t_m\rho_m/t_w\rho_w)$
† Estimated from formula $\eta_m = 1{\cdot}04\ \eta_{rel.}$

As might be expected, the viscosities and the composition percentages for bulk milk cover a narrower range than those for individual milks and, in this instance, the bulk milk values are of the same order as the lower values for individual milks.

Tapernoux & Vuillaume (16)

A Baumè capillary viscometer, similar to Oswald's, was used to determine absolute viscosity directly from the formula

$$\eta_m = kt_m\rho_m$$

Observations were made at 15°C. on samples of cow's milk after storage for 12–24 hr. at a mean temperature of 1° C. Fifteen milk samples were examined but results for two samples, one labelled mixed milk and the other abnormal, have not been used in our own analysis. The fat content of the samples was estimated by the Gerber method and density measurements, at 15° C., were taken using a density balance.

Table 3. *Viscosity and composition values obtained from* 13 *milk samples from individual cows* (16)

	Mean	Range
Viscosity (η_m cP. at 15° C.)	2·287	2·11–2·64
Fat (%)	3·80	3·15–4·80
Solids-not-fat* (%)	9·48	9·15–9·87

* Estimated from observed fat and density values.

Viscosity values and fat percentages are summarized in Table 3, together with indirect estimates of solids-not-fat percentages obtained from the observed fat and density values and standard tables (24). Although the minimum and maximum values for fat content suggest at first sight that a wide range of milks was examined, in fact, the fat percentages were between 3·6 and 3·9 for nine of the thirteen samples.

Spöttel & Gneist (9)

A Höppler rolling-ball precision viscometer was used and viscosity values were estimated from the formula

$$\eta_m = kt_b(s_b - s_m)$$

Viscosity determinations were made at 20° C. on samples of goat's milk. The total dry matter, fat and solids-not-fat percentages were reported for each sample.

Table 4. *Viscosity and composition values obtained from* 22 *samples of goat's milk* (9)

	Mean	Range
Viscosity (η_m cP. at 20° C.)	1·874	1·64–2·20
Fat (%)	2·92	2·15–3·95
Solids-not-fat (%)	8·05	6·64–9·42

The results are summarized in Table 4. The viscosity estimates are of the same order as those for cow's milk given in Tables 1, 2 and 5, in agreement with the results of other experiments by Spöttel & Gneist from which it was reported that no significant differences were found between the viscosities of cow's and goat's milk. The composition percentages given here are, in general, similar to those reported for cow's milk (Tables 2, 3 and 5) which agrees with remarks in Associates of Rogers (25) on the similarity between the average composition values of cow's and goat's milk. It seems, therefore, that these results on goat's milk may reasonably be included in the present paper, although the main concern is with cow's milk.

Seidler & Elke (4)

A Höppler rolling-ball precision viscometer was used to obtain viscosity values, the appropriate observations being made at 20° C. on samples of cow's milk. The total dry matter, fat and solids-not-fat percentages of the samples were also determined.

Detailed results were not reported for all milks, average values for total solids, solids-not-fat and viscosity being quoted for samples with the same fat percentage. Of all the samples examined twelve were found to have a fat percentage of 2·40, eleven had a fat percentage of 2·50, whilst for the remainder a particular fat percentage was generally obtained for one or two samples only.

Table 5. *Viscosity and composition values of cow's milk based on* 45 *samples and tabulated as* 18 *sets of results for publication* (4)

	Mean*	Range
Viscosity (η_m cP. at 20° C.)	2·063	1·97–2·48
Fat (%)	2·57	2·10–3·45
Solids-not-fat (%)	8·54	8·08–8·71

* Weighted according to number of samples.

The results given in Table 5 show viscosity estimates for cow's milk which appear to be generally high whilst composition percentages, particularly the fat concentrations, tend to be lower than those summarized from the experiments of Taylor and Kooper in Tables 1 and 2.

Kulkarni & Dole (22)

An Ostwald-type capillary viscometer was used and viscosity values were obtained by the formula

$$\eta_m = \frac{t_m \rho_m}{t_w \rho_w} \eta_w.$$

Experimental observations were made at 30° C. on samples of milk from goats and from Sindhi cows as soon as the samples were brought to the laboratory. The fat content of the milk samples was determined by the Gerber method, the total solids percentage gravimetrically and the solids-not-fat percentage by difference.

Table 6. *Viscosity and composition values of cow's and goat's milk* (22)

	Cow milk (individual)		Cow milk (herd)		Goat milk (individual)	
	Mean	Range	Mean	Range	Mean	Range
Viscosity (η_m cP. at 30° C.)	1·482	1·40– 1·55	1·633	1·53– 1·82	1·184	1·15–1·23
Fat (%)	4·62	4·0 – 4·9	6·66	5·4 – 7·8	3·15	2·5 –4·3
Solids-not-fat (%)	10·22	9·36–11·12	9·84	9·49–10·50	6·93	6·33–8·14
No. samples	5		5		4	

The results of those samples reported in detail are summarized in Table 6. These data exhibit some peculiarities: the viscosities for cow's milk and the composition values for bulked cow's milk are remarkably high compared with more extensive data summarized in the same paper and, further, the minimum fat percentage for bulk milk exceeds the maximum fat percentage recorded for individual cow's milk. Inquiries on these points to the authors (17. vii. 57, 18. vi. 58) have regrettably not been acknowledged. The solids-not-fat concentrations are also generally high compared with the results of Tapernoux & Vuillaume (16), Kooper (20) and Taylor (21).

RESULTS

To obtain empirical relationships for absolute viscosity (η_m) in terms of fat percentage (p) and solids-not-fat percentage (q), multiple regressions were calculated by conventional methods for all groups of data except those of Kulkarni & Dole in which there were too few observations. Following the treatment suggested by Kempthorne(26), it was found that the changes in viscosity with composition could, in general, be sufficiently represented using terms in p, q and p^2; pq and q^2 terms were in all cases unimportant ($P > 0{\cdot}10$). Regression equations of the form

$$\eta_m = A + B_1 p + B_2 q + B_3 p^2$$

have, therefore, been calculated for all groups of results. The values for the estimated partial regression coefficients, together with standard errors and the mean squares for the deviation from regression, are given in Table 7.

Table 7. *Partial regression coefficients with standard errors and significance levels, in equations* $\eta_m = A + B_1 p + B_2 q + B_3 p^2$ *connecting viscosity and the composition of milk*

Source of data	Milk type‖	Regression coefficients B_1	B_2	B_3	Deviations from regression: Mean square	D.F.
Taylor(21)	*C*, *i*	0·0419 ± 0·7606	0·1194 ± 0·0490*	0·0012 ± 0·0721	0·000703	8
Kooper(20)	*C*, *i*	−0·0303 ± 0·0649	0·1896 ± 0·0247***	0·0174 ± 0·0067*	0·002573	15
	C, *b*	0·1908 ± 0·0976†	0·1349 ± 0·0098***	−0·0079 ± 0·0159	0·000420	46
Tapernoux & Vuillaume(16)	*C*, *i*	−2·0656 ± 0·7309*	0·5380 ± 0·1330**	0·2652 ± 0·0906*	0·006467	9
Spöttel & Gneist(9)	*G*, *i*	−0·3595 ± 0·6015	0·1118 ± 0·0352**	0·0723 ± 0·0982	0·009462	18
Seidler & Elke(4)	*C*, *a*	−0·2174 ± 0·8995	0·0064 ± 0·1699	0·0990 ± 0·1602	0·014598	14
Kulkarni & Dole(22)	*C*, *i*	1·3104 ± 0·0793*	0·1307 ± 0·0027*	−0·1325 ± 0·0092*	0·000001	1
	C, *b*	−0·6468 ± 0·0959†	0·0159 ± 0·0209	0·0576 ± 0·0078†	0·000032	1
	G, *i*	−0·0265	0·0458	0·0067	0·0	0

‖ *C* = cow's milk; *G* = goat's milk; *i* = individual samples; *b* = bulk or herd milk; *a* = some published results were averages over samples.
Level of significance: † $0{\cdot}1 > P > 0{\cdot}05$; * $0{\cdot}05 > P > 0{\cdot}01$; ** $0{\cdot}01 > P > 0{\cdot}001$; *** $P < 0{\cdot}001$.

Ignoring the very limited data of Kulkarni & Dole, the mean squares due to the combined regression were significantly greater than the mean squares for deviations from regression ($P < 0{\cdot}01$ for all results except Taylor's when $P \simeq 0{\cdot}07$). Additionally, the analyses of variance showed that the proportion of the total sums of squares accounted for by the regressions exceeded 50% for each set of data examined and for over 90% in respect of Kooper's data. The unexplained variation, as indicated by the mean squares for deviation from regression, is shown in the last column of Table 7 from which the appreciable differences between workers may be noted.

The results of the regression analyses themselves indicated that viscosity was related to both the fat and the solids-not-fat concentration in the milk and, in general, viscosity was found to increase with increasing solids-not-fat concentrations for constant fat percentage and with increasing fat concentrations for a standard solids-not-fat percentage. It can be seen (Table 7) that differences among the estimated B_1 coefficients, and similarly B_3, are very marked, but for the larger sets of data, excepting Seidler & Elke and Tapernoux & Vuillaume, the estimated values of B_2 are of the same order. In view of the very small numbers of samples, the estimate of B_2 from the data of Kulkarni & Dole using samples of

individual cow's milk is in remarkably good agreement with the B_2 value obtained for the results of most of the other workers, but the value determined for goat's milk is appreciably lower and differs markedly from that obtained from the results of Spöttel & Gneist.

Table 8. *Empirical equations derived from the relationship between viscosity and milk composition (viscosity = η_mcP., fat = p%, solids-not-fat = q%)*

	Milk		Viscosity		
Source of data	Type*	No. samples	Apparatus†	Temp. (° C.)	Empirical equation $\eta_m = A + B_1p + B_2q + B_3p^2$
Taylor (21)	*C, i*	12	Cap.	20	$\eta_m = 0{\cdot}638 + 0{\cdot}0419p + 0{\cdot}1194q + 0{\cdot}0012p^2$
Kooper (20)	*C, i*	19	Cap.	*c.* 18.5	$\eta_m = 0{\cdot}038 - 0{\cdot}0303p + 0{\cdot}1896q + 0{\cdot}0174p^2$
	C, b	50	Cap.	*c.* 18·5	$\eta_m = 0{\cdot}059 + 0{\cdot}1908p + 0{\cdot}1349q - 0{\cdot}0079p^2$
Tapernoux & Vuillaume (16)	*C, i*	13	Cap.	15	$\eta_m = 1{\cdot}159 - 2{\cdot}0656p + 0{\cdot}5380q + 0{\cdot}2652p^2$
Spöttel & Gneist (9)	*G, i*	22	R.B.	20	$\eta_m = 1{\cdot}396 - 0{\cdot}3595p + 0{\cdot}1118q + 0{\cdot}0723p^2$
Seidler & Elke (4)	*C,C, a*	18	R.B.	20	$\eta_m = 1{\cdot}904 - 0{\cdot}2174p + 0{\cdot}0064q + 0{\cdot}0990p^2$
Kulkarni & Dole (22)	*i*	5	Cap.	30	$\eta_m = -3{\cdot}065 + 1{\cdot}3104p + 0{\cdot}1307q - 0{\cdot}1325p^2$
	C, b	5	Cap.	30	$\eta_m = 3{\cdot}191 - 0{\cdot}6468p + 0{\cdot}0159q + 0{\cdot}0576p^2$
	G, i	4	Cap.	30	$\eta_m = 0{\cdot}881 - 0{\cdot}0265p + 0{\cdot}0458q + 0{\cdot}0067p^2$

* See footnote to Table 7. † Cap. = capillary type, R.B. = rolling ball viscometer.

The complete set of regression equations presented in Table 8 clearly shows differences between the various workers. Further, the viscosities indicated by the constant terms in the equations being generally different from the value for the viscosity of water when $p = q = 0$ (with the exception of Tapernoux & Vuillaume), underlines the fact that these regression equations are merely empirically descriptive of the observations within the particular composition values, circumstances and experimental techniques and cannot therefore serve to give useful predictions outside these contexts.

CONCLUSIONS

Regression analyses have shown appreciable differences between the results of experimental work on the relationship between viscosity and the composition of milk and give clear indication that further experimental work is required before consistency can be obtained. These analyses have, however, given quantitative confirmation of the general knowledge that, within the ranges of composition values usually found in natural milk, the effective viscosity of milk increases with fat percentage for constant solids-not-fat percentage and increases with solids-not-fat percentage for constant fat percentage.

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ISOLATION AND IDENTIFICATION OF THE HIGH MOLECULAR WEIGHT SATURATED FATTY ACIDS OF BUTTERFAT

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Although the high molecular weight even-numbered saturated fatty acids from *n*-eicosanoic (C_{20}) to *n*-hexacosanoic (C_{26}) inclusive, have been reported as trace components of butterfat (1, 2, 3, 4) the data supporting their identifications have in most cases been meagre and inconclusive. Examination of the high molecular weight saturated fatty acids of butterfat by Bosworth & Brown (2) provided the first evidence suggesting that *n*-docosanoic (C_{22}) and *n*-tetracosanoic (C_{24}) acids were present. Similar data were obtained for *n*-octadecanoic and *n*-docosanoic acids by Bosworth & Sisson (1). Helz & Bosworth (3) also investigated these acids and, from the range of saponification equivalents obtained in the course of ester fractionation, considered that the mixture fractionated contained the even-numbered saturated components from *n*-C_{18} to *n*-C_{24}. In addition, Helz & Bosworth (3) isolated a fraction with properties akin to those of *n*-hexacosanoic acid. In all the cases referred to above, identification was based on saponification equivalents and melting points, and pure acids were not isolated. It must be stated, however, that the methyl hexacosanoate (m.p. 62·0°; sap. equiv. 410·9) prepared from the isolated *n*-hexacosanoic acid (3) corresponded closely in its properties with the pure ester (reported m.p. 63·45° (5); calculated sap. equiv. 410·6). The present work suggests that these earlier investigators presumed correctly as to the identity of the various acids, but their evidence, except in the case of methyl hexacosanoate, does not distinguish between *normal* and *iso* acids of the same carbon content. It is now known, however, that the relatively greater proportion of *normal* to *iso* acids in butterfat justifies the conclusions made.

James, Peeters & Lauryssens (6), using gas-liquid chromatography, identified *n*-octadecanoic acid in milk fat from the isolated perfused udder of a cow. In addition, they identified a series of acids which included all the *normal* odd- and even-numbered saturated fatty acids from C_7 to C_{18}. James & Martin (7) have similarly shown that *n*-octadecanoic acid occurs in goat milk fat.

The presence of *n*-eicosanoic acid in butterfat has invariably been assumed (4), but a search of the literature has failed to reveal evidence of the actual isolation and identification of this component. However, a recent gas-liquid chromatographic analysis of New Zealand butterfat by Hawke (8) indicated that both *n*-eicosanoic acid and *n*-octadecanoic acid were present.

In the *normal* odd-numbered series, *n*-undecanoic (C_{11}) (9) *n*-tridecanoic (C_{13}) (10) *n*-pentadecanoic acid (C_{15}) (11) and *n*-heptadecanoic acid (C_{17}) (12), have been isolated from butterfat, but the higher members have not hitherto been established as components of milk fat although *n*-nonadecanoic acid (C_{19}) (13), *n*-heneicosanoic acid (C_{21}) (14) and

n-tricosanoic acid (C_{23})(14) have recently been isolated from hydrogenated ox perinephric fat.

The purpose of this present investigation was to isolate and identify in butterfat *n*-octadecanoic and *n*-eicosanoic acids as well as those *n*-odd-numbered acids of higher molecular weight than *n*-heptadecanoic acid, and to confirm by physical and chemical constants earlier work which suggested the presence of *n*-docosanoic (C_{22}), *n*-tetracosanoic (C_{24}) and *n*-hexacosanoic (C_{26}) acids.

EXPERIMENTAL

For the isolation of *n*-C_{20} and higher saturated fatty acids the solid residue (SR) fractions from seventeen earlier analyses, which had been carried out to determine seasonal variations in the fatty acid composition of New Zealand butterfat(15), were bulked and used. Typical of the SR methyl ester fractions when freed from unsaponifiable matter, were D/12, 3 SR (sap. equiv. 303·4, iodine value 10·7) and C56, 1, SR (sap. equiv. 310·1, iodine value 10·6). Low-temperature crystallization from solvents of this composite methyl ester sample (wt. 270·0 g.), first as fatty acids, and subsequently as methyl esters, finally yielded from 30 volumes ethyl ether at −40°, an insoluble fraction (wt. 206·0 g., m.p. 35·8–36·4°), which was fractionated in a type G column(11). Refractionation in a type E column(16) of the resulting residue, followed by repeated low temperature crystallization yielded the high molecular weight fatty acids now reported.

For the isolation and identification of *n*-octadecanoic acid, the fraction used was C/38, 1, S9, wt. 34·97 g., which also resulted from an earlier ester fractionation analysis(15) made to determine seasonal variations in fatty acid composition. Its properties were as follows: sap. equiv. (methyl esters) 297·5; iodine value 8·1.

Purification was effected by means of seven crystallizations, the first two from 40 volumes acetone at −35°, the next three from 40 volumes ethyl ether at −35° and the final two from 10 volumes acetone at room temperature.

RESULTS

n-Octadecanoic acid (*stearic acid*, C_{18})

Fraction AS6S (wt. 16·5 g.). M.p. 69·9–70·2° (reported m.p. 69·7°, 69·6°(17, 5)); when mixed with an equal quantity of pure *n*-octadecanoic acid (m.p. 69·7–69·8°) gave m.p. 69·6–69·8°; sap. equiv. 284·2 (calc. for $C_{18}H_{36}O_2$, 284·5); combustion analysis, C 76·0%, H 12·7% (calc. for $C_{18}H_{36}O_2$, C 76·0%, H 12·8%); iodine value 0·0; X-ray long spacing 39·05Å (a sample of pure *n*-octadecanoic acid gave 39·05Å; reported values 40·0 Å, 39·75 Å(17, 5)); n_D^{75} 1·4317 (reported value n_D^{75} 1·4318(18)); methyl ester: m.p. 38·2–38·8° (reported, 38·7(5)); n_D^{45} methyl ester 1·4349 (reported n_D^{45} 1·4346(19)).

n-Nonadecanoic acid (C_{19})

Fraction B7S4LS (wt. 0·11 g.). M.p. 66·5–66·7° (reported m.p. 68·5°, 68·65°(17, 5)); X-ray long spacing 43·7 Å when crystallized from benzene (reported value 44·7 Å(17)); combustion analysis, C 76·6%, H 12·7% (calc. for $C_{19}H_{38}O_2$, C 76·5%, H 12·8%).

When analysed by gas-liquid chromatography (thermal conductivity apparatus(20), this fraction was found to contain the following components: *n*-C_{18}, 7·4%; *n*-C_{19}, 67·6%; *n*-C_{20}, 25·0% (expressed in moles %).

n-Eicosanoic acid (*Arachidic*, C_{20})

Fraction B2S6S (wt. 2·80 g.). M.p. 74·8–75·3° (reported m.p. 75·35°, 75·1° (5, 17)), when mixed in equal proportions with pure *n*-eicosanoic acid (m.p. 74·9–75·3°) gave m.p. 74·9–75·4°; methyl ester m.p. 45·5–46·0° (recorded m.p. 46·6° (5)); sap. equiv. 311·8 (calc. $C_{20}H_{40}O_2$, 312·5); combustion analysis, C 76·6%, H 12·7% (calc. for $C_{20}H_{40}O_2$, C 76·8%, H 12·9%); X-ray long spacing 43·3 Å (reported values 43·9 Å, 44·15 Å (17, 5)).

n-Heneicosanoic acid (C_{21})

Fraction B16S3S. (wt. 0·58 g.). M.p. 74·7–75·3° (reported m.p. 74·8°, 74·3° (17, 5)); when mixed in equal proportions with *n*-heneicosanoic acid (U124S4S, m.p. 72·3–72·7° (14)) gave m.p. 73·7–74·6°; sap. equiv. 324·8 (calc. for $C_{21}H_{42}O_2$, 326·6); combustion analysis, C 77·4%, H 12·8% (calc. for $C_{21}H_{42}O_2$, C 77·2%, H 13·0%); iodine value 0·0; X-ray long spacing when crystallized from petroleum ether b.p. 90°, 48·6 Å (reported values for the B′ crystal form, 49·1 Å, 49·25 Å (17, 5)).

Fraction B17S6S (wt. 0·23 g.). M.p. 73·3–73·7°; when mixed in equal proportions with *n*-heneicosanoic acid (U124S4S, m.p. 72·3–72·7° (14)) gave m.p. 73·1–73·6°; X-ray long spacing when crystallized from petroleum ether (b.p. 90°) 48·6 Å.

n-Docosanoic acid (*Behenic*, C_{22})

Fraction B4S19S (wt. 1·17 g.). M.p. 79·0–79·2° (reported m.p. 79·9°, 79·95° (17, 5)); when mixed with an equal quantity of pure *n*-docosanoic acid (U121S5S m.p. 78·9–79·1° (14)) gave m.p. 79·0–79·2°; methyl ester m.p. 51·3–51·5° (reported m.p. 52·7° (5)); sap. equiv. 340·7 (calc. for $C_{22}H_{44}O_2$, 340·6); combustion analysis, C 77·7%, H 13·0% (calc. for $C_{22}H_{44}O_2$, C 77·6%, H, 13·0%); iodine value 0·0; X-ray long spacing 47·7 Å (reported value for *n*-C_{22}, 48·3 Å (17, 5)).

Fraction B25S5S (wt. 0·61 g.). M.p. 79·6–80·6°; when mixed in equal proportions with behenic acid (fraction U121S5S, m.p. 78·9–79·6° (14)) gave m.p. 79·0–79·7°; sap. equiv. 339·2; combustion analysis, C 77·9%, H 13·1%; iodine value 0·0; X-ray long spacing 48·8 Å.

n-Tricosanoic acid (C_{23})

Fraction B5S17LS (wt. 0·24 g.). M.p. 78·0–78·2 (reported m.p. 79·1° (5, 17)); when mixed with an equal quantity of *n*-tricosanoic acid from ox fat (U120S5S, m.p. 78·3–78·8° (14)) gave m.p. 78·2–78·5°; sap. equiv. 357·7 (calc. for $C_{23}H_{46}O_2$, 354·6); combustion analysis, C 77·9%, H 13·1% (calc. for $C_{23}H_{46}O_2$, C 77·9%, H 13·1%); iodine value 0·0; X-ray long spacing 54·4 Å. (reported values for *n*-tricosanoic acid 53·6 Å., 53·4 Å. (17, 5)).

n-Tetracosanoic acid (*Lignoceric* C_{24})

Fraction B10S2S (wt. 1·24 g.). M.p. 84·1–84·3° (reported m.p. 83·9°, 84·15° (17, 5)); sap. equiv. 368·4 (calc. for $C_{24}H_{48}O_2$, 368·6); combustion analysis, C 78·4%, H 13·1% (calc. for $C_{24}H_{48}O_2$, C 78·2%, H 13·1%); iodine value 0·0; X-ray long spacing 51·8 Å (reported values 52·6 Å, 52·9 Å (5, 17)).

Fraction B12S4S (wt. 0·25 g.). M.p. 83·1–83·6°; sap. equiv. 368·5 when mixed in equal quantity with fraction B10S2S reported above, gave m.p. 84·0–84·6°.

n-Hexacosanoic acid (Cerotic, C_{26})

Fraction B19S9S (wt. 0·55 g.). M.p. 87·3–87·9° (reported m.p. 87·5°, 87·7°(17, 5)); when mixed with an equal quantity of *n*-hexacosanoic acid from ox fat (U125S12S m.p. 85·7–86·4°(14)) gave m.p. 86·8–87·3°; sap. equiv. 397·7 (calc. for $C_{26}H_{52}O_2$, 396·7); combustion analysis, C 78·8%, H 13·1% (calc. for $C_{26}H_{52}O_2$, C 78·7%, H 13·2%); iodine value 0·8; X-ray long spacing 56·8 Å (reported values 57·1 Å, 56·25 Å (17, 5)); m.p. ethyl ester 60·0–60·2° (reported m.p. 60·2°(5)).

Fraction B20S4S (wt. 0·33 g.). M.p. 86·5–86·7°; when mixed with an equal quantity of *n*-hexacosanoic acid from ox fat (U125S12S, m.p. 85·7–86·4°(14)) gave m.p. 86·1–86·3°; sap. equiv. 398·7; combustion analysis, C 79·1%, H 13·3%; iodine value 0·0; X-ray long spacing for methyl ester 65·5 Å (reported value for methyl ester 66·15 Å(5)); m.p. methyl ester 62·0–62·4° (reported m.p. 63·45°(5)).

Melting points were determined in closed capillaries and are uncorrected.

X-ray measurements were made with a Philips Geiger X-ray spectrometer using manganese-filtered $K\alpha$ radiation. In the preparation of samples for X-ray analyses, samples of even numbered acids were melted on a glass slide and cooled, while in the case of odd-numbered acids slides were prepared by crystallization from light petroleum (b.p. 40–60° for *n*-C_{19}, and b.p. 100–120° for *n*-C_{21} and *n*-C_{23}).

Combustion analyses were determined by Dr K. W. Zimmermann, Organic Microanalytical Laboratory, Division of Industrial Chemistry, C.S.I.R.O., Melbourne, Australia.

DISCUSSION

The published results of fatty acid composition analyses of butterfat(4, 15) invariably indicate the presence of higher (above C_{18}) saturated fatty acids, but owing to the amounts present being small, it had not been practicable to separate these fatty acid components. The customary procedure for determining the amounts of higher saturated acids is to assume that the saturated acids of the residual 'solids' consist of a mixture of stearic and arachidic acids, and to calculate those acids of higher molecular weight than stearic acid as arachidic acid. In this way the amounts of higher saturated acids in butterfat have usually been found to fall within the range of 0·5–1·0 mol.% of the total fatty acids(4, 15). As acids of higher molecular weight than arachidic are present, the reported amounts of higher saturated fatty acids normally calculated as arachidic acid must be considerably in excess of those actually present, and the amount of stearic acid as calculated is accordingly less than what is actually present. In this investigation we have not made quantitative estimates of the amounts of individual higher saturated acids present in the numerous fractions as the crystallization procedures for the preparation of pure constituents inevitably preclude quantitative recovery. However, we have been able to establish the presence in butterfat of *n*-eicosanoic, *n*-heneicosanoic, *n*-docosanoic, *n*-tricosanoic and *n*-tetracosanoic acids, and have confirmed the presence of *n*-hexacosanoic acid. Furthermore, pure acids have been isolated of which the physical and chemical properties have provided conclusive proof of identity. The approximate percentages of these high molecular weight components have been determined independently by gas-liquid chromatographic analyses of a 'solid' residue sample (C/38, 2, SR(15) excluding unsaponifiable matter) which was representative of the starting material in this experiment. Expressed as weight percentages of the total fatty acids, the following proportions

were found to be present: *n*-C_{20}, 0·21%; *n*-C_{21}, 0·05%; *n*-C_{22}, 0·07%; *n*-C_{23}, 0·06%; *n*-C_{24}, 0·05%; *n*-C_{26}, 0·06%.

From the results of other investigators, based on melting-points and saponification equivalents alone, it was not feasible to exclude the possibility that the constituents separated were *iso* and not *normal* acids, as the melting-points of *iso* acids are generally less than 1° below the melting-points of the corresponding *normal* acids (21). In this present work melting-points and saponification equivalents have been supported by mixed melting-points, X-ray long spacings, iodine values and combustion analyses, and it has been conclusively established that these acids belong to the *normal* series and not the *iso*. The higher *iso* acids, if present, must form a relatively small proportion of the original 'solid' residue.

Trace quantities of the *normal* odd-numbered higher acids have now been isolated from butterfat for the first time. The *n*-C_{21} and *n*-C_{23} components were isolated in pure form, while the *n*-C_{19} component contained small amounts of adjacent even-numbered fatty acids, the presence of which was shown by gas-liquid chromatography. In all cases the chemical and physical properties confirmed the identity of the *n*-C_{19}, *n*-C_{21} and *n*-C_{23} constituents. As is characteristic of the *normal* odd-numbered acids, these constituents all shrank from the wall of their containing vessels on solidification.

With the identification of these high molecular weight acids, the fatty acid composition of butterfat is further elucidated and earlier knowledge of the constituents of milk fat is extended by actual isolation of pure acids, so that it now includes all the odd- and even-numbered *normal* saturated acids from *n*-C_{10} to *n*-C_{26}, except *n*-C_{25}. This latter acid is probably present in trace amounts, but has eluded isolation by the methods employed.

The higher even-numbered saturated fatty acids of butterfat (*n*-C_{20} to *n*-C_{26}) are reported by Helz & Bosworth (3) to consist mainly of *n*-hexacosanoic acid. These authors estimate that more than half of the weight of the high molecular weight acids, representing about 0·25% of the total fatty acids (22), comprise the *n*-C_{26} component. From the work of Pollard, Chibnall & Piper (23), and Smith & Chibnall (24), these higher acids appear to be present in pasture lipids also, where a considerable proportion of the weight is *n*-hexacosanoic acid, and where they constitute about 0·1% of the dry matter. Accordingly, it would appear that the presence in butterfat and in ox perinephric fat (14) of these even-numbered high molecular weight saturated acids, is the result of direct assimilation of dietary lipids as postulated by Hilditch (4).

SUMMARY

Butterfat has been shown to contain the *normal* odd-numbered saturated acids *n*-nonadecanoic acid (C_{19}), *n*-heneicosanoic acid (C_{21}), and *n*-tricosanoic acids (C_{23}).

The presence of the normal *even*-numbered acids *n*-octadecanoic acid (C_{18}), *n*-docosanoic acid (C_{22}), *n*-tetracosanoic acid (C_{24}) and *n*-hexacosanoic acid (C_{26}) is conclusively established.

n-Eicosanoic acid (C_{20}) formerly assumed to be present in butterfat has been isolated and identified by its physical and chemical properties.

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OESTROGENS IN BRITISH PASTURE PLANTS

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The detection of oestrogens by bioassay in a few of the plants found in British pastures has already been reported (1, 2), and in the case of *Trifolium pratense* (red clover) the active substances concerned have been isolated and found to be the very weakly oestrogenic isoflavones biochanin A and genistein (3, 4). In addition, a coumarin of novel chemical structure has recently been isolated from *T. repens* (white clover, Ladino variety) and found to have oestrogenic potency about 30 times greater than genistein or biochanin A (5, 6, 7). The new coumarin, named coumestrol, is presumably also present in varieties of *T. repens* occurring in British pastures. The immediate interest in these oestrogens of pasture plant origin arises from the discoveries of Folley (8) and Folley, Scott-Watson & Bottomley (9) that oestrogens administered to lactating cows can cause changes in milk composition, in particular an increase of the percentage of non-fatty solids in the milk which may persist for several weeks. These findings have recently been confirmed and placed on a more quantitative basis by Hutton (10).

Some further interest is also attached to pasture plant oestrogens because of the known pathological effect on the breeding of sheep owing to excessive oestrogen stimulation (11, 12, 13, 14, 15) which may follow upon high intake of *T. subterraneum* (subterranean clover), a plant which has also been found to contain genistein and biochanin A (16, 17, 18, 4). In addition, oestrogens in pasture plants might also be of interest in view of the known important effects of oestrogen on growth in cattle and sheep for meat production.

A more intensive survey of the oestrogenic activity of about twenty species commonly found in temporary and permanent pastures in Great Britain was therefore undertaken. In most cases plants were sampled on three occasions during the growing season, so that some information on the change in oestrogen content in relation to time within a growing season could be obtained.

MATERIALS AND METHODS

Collection of plant specimens

Samples of plant material for assay were taken from several varieties of *Medicago sativa* (lucerne), from three other species of *Medicago*, from several *Trifolium* species and from about twelve grass species. The *M. sativa* varieties were each sampled once only (in late October 1953), but most of the other plants were sampled three times during the spring-summer growing season of 1954.

The *M. sativa* samples were taken from plants growing at the Grassland Research Institute, Hurley; the *Lolium italicum* × *L. perenne* (short rotation ryegrass) sample was

taken from the Grassland Research Institute, Drayton. All other samples were taken from plants growing in the Reading University Agricultural Botany Garden, Shinfield, or from National Institute for Research in Dairying farms at Shinfield.

Method of sampling

The species (or variety of a species) was sampled by cutting material from the aerial parts of several individuals. From this, 100 g. of fresh plant material was taken for assay, care being taken that the proportions of leaf, stem, flower-head, etc., in the sample were, as far as could be judged, the same as in the growing plants. In a few cases the final sample for assay was less than 100 g. owing to shortage of plant material. In some cases (noted in the tables of results) leaf-blades, stem and flower-heads were separately sampled. The term 'stem' is here applied to the remainder of the aerial portion of the plant other than leaf-blades and flower-heads.

Choice of assay procedure

Bioassay, rather than chemical assay, of the oestrogens in the plant samples was employed, since it could be assumed that the former method would give an estimate of total oestrogenic activity, whether this was due to the isoflavones known to occur in *Trifolium pratense* or to unknown oestrogens.

Several satisfactory oestrogen bioassay methods were available including that based on uterine growth in the immature mouse, which was the method of assay used in earlier work on pasture oestrogens (19, 2), the Allen-Doisy rat vaginal cornification method (20, 21), and also a method based on uterine growth in the guinea-pig.

In using mouse or rat bioassays for detecting oestrogen in plant samples the most practicable method of administration is by subcutaneous injection, and experience has shown that the immature mouse, for example, can tolerate a maximum dose of only about 20 mg. of most plant extracts. It is thus essential in using these test animals to effect considerable concentration of the oestrogens in the plant material. This involves solvent extraction of the plant sample and partial separation of the many substances in the extract to yield a number of fractions. Often a rather arbitrary decision has to be taken, based on knowledge of physical properties and chemistry of known oestrogens, as to which fractions it is worthwhile submitting to the bioassay. Use of the guinea-pig assay in which the animals are fed exclusively on the plant sample to be tested avoids these difficulties, but it is a lengthy and expensive procedure and could not be used where a considerable number of samples were to be assayed.

The immature mouse uterine weight assay was chosen for our work; it has an approximately 20-fold greater sensitivity than the rat vaginal cornification method.

Extraction of samples for bioassay

Reported methods (19, 1, 2) for extracting plant samples and fractionating the extracts involve treating the crude plant extracts with hot sodium hydroxide solutions. This procedure permits the ready separation of fats, waxes and chlorophyll as water-soluble sodium salts from non-saponifiable lipids. The use of strong alkali is, however, objectionable in view of the fact that other plant constituents, e.g. flavonoids, also undergo chemical change under alkaline conditions; and when the oestrogenic isoflavone, genistein, was isolated by Bradbury & White (16) from *T. subterraneum* it was clear that strong alkali

could no longer be used in the extraction of oestrogens from plant material. The method described below was therefore devised to avoid its use.

The sample (100 g.) was as quickly as possible after cutting (usually within 1 hr. but always within 4 hr.) minced in 95% ethanol (500 ml. or rather less if convenient) using a Townson and Mercer top-drive macerator, the resulting mixture being then refluxed for 0·5 hr., cooled, filtered and the residue re-extracted with boiling 95% ethanol (200 ml.) for 0·5 hr. The filtrates were combined, cooled and brought to 70% (v/v) ethanol (specific gravity 0·89 at 20° C.) by adding water. Then 0·5 volume of light petroleum (boiling range 60–80° C.) was added to the aqueous ethanol solution and the two phases well shaken together and allowed to separate. The top phase was discarded and the lower phase distilled to dryness at a pressure of about 20 mm. mercury. Water (100 ml.) was added to the residue and the mixture extracted with 5 × 100 ml. ether. The ether and the water solutions were then processed separately.

(*a*) *Ether solution*

This solution was dried with Na_2SO_4, evaporated to dryness, and the residue dissolved in methanol (80 ml.), water (20 ml.), light petroleum boiling range 60–80° C. (50 ml.). The two phases were thoroughly shaken together and then allowed to separate. The lower and upper phases were then evaporated to dryness at about 20 mm. mercury pressure yielding two fractions for assay (F1 and F2 respectively).

(*b*) *Water solution*

To the water phase was added 0·1 volume of 28% (v/v) sulphuric acid in water and it was then refluxed for 0·5 hr. It was then cooled, brought to pH 7 by cautious addition of solid $NaHCO_3$ and extracted with 2 volumes of *n*-butanol in portions. The *n*-butanol solution was then dried with Na_2SO_4, evaporated to dryness (finally at about 1 mm. mercury pressure) and the residue dissolved in ether (200 ml.) and water (50 ml.). The two phases were well shaken together, allowed to separate and the ether phase separated, dried with Na_2SO_4 and evaporated to dryness yielding the third fraction for assay (F3).

In the procedures described solvents and reagents were of good laboratory reagent grade, care being taken to use peroxide-free ether.

Bioassay procedure

The whole of each of the F1, F2 and F3 fractions from each plant sample was then submitted to the following bioassay, except where a fraction exceeded 200 mg., in which case an approximately 200 mg. portion was taken. The portions of the F1 and F3 fractions for assay were each dissolved in 3 ml. 50% (v/v) propylene glycol in water and that of the F2 fraction in 3 ml. arachis oil. Each 3 ml. solution of unknown oestrogen content provided six 0·3 ml. mouse doses for the following procedure and 1·2 ml. spare solution.

The mouse uterine weight method was used as a weekly routine. Immature mice of body weight 8–10 g. were received from the breeding colony of the Agricultural Research Council Field Station, Compton, on the Tuesday morning and then placed randomly into the following groups each of six animals: one group to serve as controls; two groups for two dose levels of a standard oestrogen, oestradiol-17β; and the remaining groups for unknowns, one group per unknown solution. Doses of 0·02 and 0·04 μg. oestradiol-17β per mouse were used in the two standard oestrogen groups. Each mouse received its dose

of 0·3 ml. solution in portions of 0·05 ml. solution twice daily for three consecutive days. The oestradiol-17β was dissolved in 50% (v/v) propylene glycol in water and this solvent mixture was also used for the control group. Mice were killed on the Friday morning and the uteri dissected out and placed in Bouin's fluid. On the following Monday the uteri were pressed dry on blotting-paper, weighed, and the mean uterine weight and standard deviation for each group were then calculated.

By comparing the response given by an unknown solution with the responses given by the standard doses of oestradiol-17β an approximate estimate of the quantity of oestradiol-17β needed to give the response of the unknown could be made. When the estimate was less than 0·005 μg. it was considered that the unknown had no oestrogenic activity.

RESULTS

The three fractions (F1, F2, F3) obtained from a sample were each bioassayed at a single dose level, expressed in the following tables as weight (g.) of fresh plant matter per mouse. Thus, for example, if 100% of each of the F1, F2 and F3 fractions from a 100 g. sample of plant material had been assayed, doses would be expressed in each case as 10 g. fresh plant matter per mouse, there being altogether 10 mouse doses in each 3 ml. solution prepared for assay.

Table 1. *Oestrogenic activity of varieties of* M. sativa (*lucerne*)

Variety	Date of cutting	Dose (g. fresh plant per mouse) F1	F2	F3	Response (μg. oestradiol-17β) F1	F2	F3
Welsh (S205)	20. x. 53	2·5,	5·9,	—	0·015,	0,	—
Provence (stem)	23. x. 53	10·0,	5·0,	4·0	0·007,	0,	0
Ile de France (leaf)	23. x. 53	3·0,	3·0,	3·0	0·008,	0,	0
Dupuis (stem)	23. x. 53	6·0,	5·0,	2·5	0·012,	0,	0
Dupuis (leaf)	23. x. 53	3·0,	3·0,	2·5	0·008,	0,	0

In addition to the results of Table 1 the following samples of *M. sativa* varieties also collected on 23. x. 53 were tested at the doses (F1, F2 and F3 fractions) indicated and found to have no activity: Old Franconian (leaf) 5·0, 2·0, 3·0; Old Franconian (stem) 5·0, 10·0, 2·0; Provence, Marais type (stem) 5·0, 10·0, 13·0; Provence, Marais type (leaf) 4·0,

Table 2. *Oestrogenic activity of* Medicago *species other than* M. sativa

Plant material tested	Date of cutting	Dose (g. fresh plant per mouse) F1	F2	F3	Response (μg. oestradiol-17β) F1	F2	F3
M. lupulina (Black medick)							
leaf	8. vii. 53	0·75,	—	1·5	0·005,	—	0
stem	8. vii. 53	1·5,	—	1·25	0·008,	—	0
flowerheads	8. vii. 53	1·5,	—	0·7	0,	—	0
M. falcata (Sickle lucerne)	20. x. 53	1·9,	2·9,	—	0·006,	0,	—
M. media (Sand lucerne)	20. x. 53	3·3,	—	—	0·006,	—	—

Table 3. *Oestrogenic activity of* Trifolium *species*

Plant material tested	Date of cutting	Dose (g. fresh plant per mouse) (F1, F2 or F3)	Response (μg. oestradiol-17β) (F1, F2 or F3)
T. repens (white clover), strain S100	23. iv. 54		
	14. vi. 54	10·0 (F3)	0·008
	22. vi. 54	10·0 (F1)	0·013
T. repens, Kent wild white	14. v. 54		
	10. vi. 54		
	7. vii. 54	10·0 (F1)	0·021
T. repens, New Zealand strain	15. iv. 54	10·0 (F1)	0·006
	1. vi. 54		
	7. vii. 54	10·0 (F1), 10·0 (F3)	0·015, 0·018
T. pratense (red clover), strain S151	30. iii. 54	10·0 (F3)	>0·04
	7. vii. 54	10·0 (F3)	0·008
	11. viii. 54	10·0 (F2)	0·012
	1. ix. 54		
T. pratense, strain S151: leaf	30. vii. 54	3·0 (F1), 3·0 (F2)	0·015, 0·006
	7. ix. 54		
T. pratense, strain S151: stem	30. vii. 54		
	7. ix. 54		
T. pratense, strain S151: flowerheads	7. ix. 54	2·8 (F1)	0·019
T. hybridium (alsike clover)			
Leaf	30. vii. 54	8·0 (F2)	0·005
Stem	30. vii. 54	7·0 (F1)	0·012
Flowerheads	30. vii. 54	4·8 (F2)	0·005
T. incarnatum (crimson clover)	15. iv. 54	3·0 (F1)	0·006
	14. v. 54	10·0 (F3)	0·047
	3. vi. 54		

Where no details of fractions are given, no activity was detected.

Table 4. *Oestrogenic activity of grasses*

Species	Date of cutting	Dose (g. fresh plant per mouse) (F1, F2 or F3)	Response (μg. oestradiol-17β) (F1, F2 or F3)
Agrostis tenuis (common bent)	22. iv. 54	10·0 (F1)	0·006
	1. vi. 54	10·0 (F1)	0·014
	23. vii. 54		
Dactylis glomerata (cocksfoot), strain S143	8. iv. 54	10·0 (F3)	0·005
	14. iv. 54		
	3. vi. 54		
	22. vi. 54		
Festuca elatior pratensis (meadow fescue), strain S215	12. iv. 54	10·0 (F3)	0·008
	14. v. 54		
	4. vi. 54		
	1 vii. 54		
Festuca rubra var. *arenaria* (red fescue)	14. iv. 54		
	1. vi. 54		
	7. vii. 54	10·0 (F1)	0·005
Lolium italicum × *L. perenne* (short rotation ryegrass)	14. iv. 54	10·0 (F1)	0·01
Poa trivialis (rough-stalked meadow grass)	14. v. 54		
	3. vi. 54	5·0 (F2)	0·01
	22. vii. 54	10·0 (F1)	0·005

Where no details of fractions are given, no activity was detected.

3·0, 2·5; Provence (leaf) 3·0, 3·0, 3·0; Alexander's strain (stem) 6·0, 10·0, 3·0; Alexander's strain (leaf) 2·5, 3·0, 4·0; Ile de France (stem) 3·0, 10·0, 3·0.

Trifolium fragiferum (strawberry clover) was sampled on 23. x. 53 and *Trifolium dubium* (suckling clover) on 13. v. 54, 14. vi. 54 and 29. vi. 54 but no activity found in either case.

The following grasses were also sampled on the dates indicated but no activity found: *Agropyron repens* (couch grass) 14. iv. 54, 1. vi. 54, 21. vii. 54; *Alopecurus pratensis* (meadow foxtail) 16. vi. 54; *Bromus inermis* (awnless brome grass) 12. iv. 54, 10. vi. 54, 7. vii. 54, 13. vii. 54; *Cynosurus cristatus* (crested dog's tail) 17. vi. 54; *Holcus lanatus* (Yorkshire fog) 13. iv. 54, 28. v. 54, 29. vi. 54; *Lolium italicum* var. giant evergreen (Italian ryegrass) 25. iii. 54, 13. v. 54, 14. vi. 54, 1. vii. 54; *L. perenne* var. Ayrshire (perennial ryegrass) 8. iv. 54, 14. v. 54, 4. vi. 54; *Phleum pratense* (Timothy grass) 9. iv. 54, 28. v. 54, 4. vi. 54.

DISCUSSION

It may be seen from the data given that most of the leguminous plants and about half of the grasses commonly found in British pastures contain detectable quantities of oestrogen. From the results with the *Trifolium* species and grasses tested on more than one occasion, it appears that the quantity of oestrogen present is not constant during the growing season. The three varieties of *T. repens*, for instance, all appear to have a maximum oestrogen content around flowering time (end of June), but it would be necessary to test considerably larger samples to confirm this. A further interesting feature of the results is the rather high activity of the F3 fraction shown on one occasion each by *T. pratense* and *T. incarnatum*, suggesting the presence in these plants of a water-soluble conjugate of an oestrogen from which the oestrogen is liberated by acid hydrolysis, a known example being the glucoside of genistein found in soya bean (22).

In attempting to assess whether there is sufficient oestrogen in pasture plants in Great Britain to influence milk composition in the cow, one may first consider Hutton's finding (10) that an injection of 12·5 mg. of oestradiol-3-benzoate (as a microcrystalline suspension) will generally give marked changes in milk composition in the lactating cow. He concluded that absorption of the injected crystals took place over a period of 5–6 weeks and that oestrogen, presumably as the free oestradiol-17β, would be liberated into the circulation at a rate of 300–400 μg. per day. It is known that a cow will consume as much as 50 kg. plant material per day from pastures and thus, for this to provide sufficient oestrogen to affect milk composition, each gramme of ingested material would have to result in the addition to the circulation of a quantity of oestrogen equal in its effect on milk composition to 0·006–0·008 μg. circulating oestradiol-17β. From our comparisons of pasture plant extracts with oestradiol-17β in the immature mouse uterine weight test, it seems that for cows pastured even on the more oestrogenic species, the possibility of so great an addition is unlikely. This cannot however be entirely ruled out.

Owing to the very low potency of known pasture oestrogens and the high cost of their preparation in large quantities, the administration of pure compounds to lactating cows is at present impracticable. Experiments in which oestrogenic pasture species were cut and fed in measured quantities to lactating cows might, however, serve to show whether the oestrogens are present in sufficient quantity to affect milk composition.

SUMMARY

1. Four species of *Medicago*, six species of *Trifolium* and fourteen grass species, all frequently found in British dairy pastures, have been tested for oestrogenic activity by the mouse uterine weight method. Activity was detected in four, four and six species respectively.

2. It is probable that the quantity of oestrogen present in British pasture plants is insufficient to affect milk composition in cows at pasture. Some further experiments with cows are, however, suggested which might elucidate this.

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THE EFFECT OF HEAT ON THE VITAMIN B_6 OF MILK

I. MICROBIOLOGICAL TESTS

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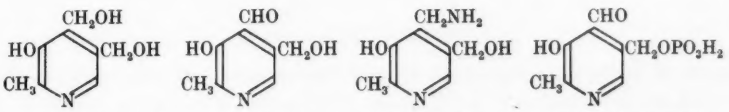
(With 3 Figures)

Vitamin B_6 occurs naturally in several different forms, pyridoxine, pryidoxal, pyridoxamine and their 5′ phosphates. The three analogues differ by having either a hydroxymethyl, aldehyde or aminomethyl group in the 4 position:



Pyridoxine Pyridoxal Pyridoxamine Pyridoxal phosphate

Because of these different reactive groups, the analogues do not always behave in the same way in chemical, microbiological and biological tests, and consequently the measurement of vitamin B_6 activity in natural materials is not straightforward. Under standard conditions the three analogues are about equally active in growth tests for chicks and rats (1) and therefore the total vitamin B_6 activity of foodstuffs for animals is probably most accurately estimated by biological tests. However, microbiological methods are quicker and often more convenient to use. Moreover, some indication of the forms of the vitamin present in the test materials can be obtained by the use of several micro-organisms having different requirements for the vitamin B_6 analogues.

We have recently been investigating in this laboratory the loss of vitamin B_6 activity in the heat treatment of milk. Vitamin B_6 was estimated by biological and microbiological methods. As part of this investigation various microbiological methods for measuring vitamin B_6 were studied in some detail. The procedures finally adopted as being satisfactory with milk are described in this paper. In addition, the differential assay method of Rabinowitz & Snell (2) has been used to determine the forms of the vitamin (i.e. pyridoxine, pyridoxamine or pyridoxal) that predominate in raw milk and in evaporated milk. Results obtained by the original method of Rabinowitz & Snell (2) and by a modified method using *Streptococcus faecium* Ø51 instead of *Str. faecalis* R are presented. They confirm the findings of Hodson (3) that pyridoxal predominates in raw milk, whereas a large proportion of the vitamin B_6 activity of heat-treated milk is due to pyridoxamine. Further tests that give support to these findings are also described.

EXPERIMENTAL

Milk samples

Raw milk, evaporated milk and evaporated milk stored for 6 months at room temperature, all from the same batch of original milk, were freeze-dried for biological and microbiological tests as described in the companion paper (4). The evaporated milk was canned and sterilized at 240° F. for 20 min.

Samples of evaporated and sweetened-condensed milks (prepared by different manufacturers) and of sterilized whole milk were purchased from a local grocer. These milks were compared with samples of evaporated milk which had been stored at the N.I.R.D. for 2 years at room temperature, and with a sample of sterilized whole milk which had been similarly stored for $6\frac{1}{2}$ years. A bulk sample of human milk was obtained from Battle Hospital, Reading, and bulk samples of goat's milk and sow's milk from the Institute herds.

The liquid milk samples were deep-frozen as soon as possible after collection. The freeze-dried milks were stored in air-tight bottles in a refrigerator.

Treatment of samples

Extraction of the vitamin B_6 activity of milk by treatment with acid. Liquid whole milk (2 ml.) or whole dried milk (0·5 g.) in 90 ml. 0·055 N-HCl were either heated in an autoclave at 120° C. for periods of from 30 min. to 4 hr. or heated in steam for 30 min. After cooling, the volumes were made up with water to 100 ml., the solutions filtered at pH 4·6 and the clear filtrates diluted to the appropriate concentrations for assay with the different test organisms (see Microbiological methods).

Inactivation of pyridoxamine with nitrous acid. The method of Snell (5) was used. To 0·5 g. of a freeze-dried milk sample in 10 ml. water were added 0·25 ml. glacial acetic acid and 10 mg. $NaNO_2$ in 1 ml. of water. The mixture was shaken for 30 min. at 30° C. Then 25 mg. urea were added and the shaking continued for a further 30 min. The reaction mixtures were diluted with water to about 100 ml. and heated in steam for 30 min. After cooling the solutions were made to a volume of 100 ml. and filtered at pH 4·6. The clear filtrates were then diluted appropriately for assay with the various test organisms (see Microbiological methods). Pyridoxal, pyridoxamine and pyridoxine in 5 μg. quantities were treated similarly.

Inactivation of pyridoxal with alkali and acetone. Again the method of Snell (5) was used. Samples of freeze-dried milk (0·5 g. in 5 ml. water) were kept at 37° C. for 2 hr., with 2 ml. N-NaOH and 1 ml. acetone. The volume was then made up to about 90 ml., 7 ml. N-HCl were added and the mixture was heated in steam for 30 min. After cooling the solution was made up to a volume of 100 ml. at pH 4·6, filtered and diluted to the appropriate concentration for addition to the assay tubes as described later (see Microbiological methods). Pyridoxal, pyridoxamine and pyridoxine in 5 μg. quantities were treated similarly.

Standard solutions of vitamin B_6

Stock solutions of pyridoxine hydrochloride and pyridoxamine dihydrochloride (Roche Products Ltd) and of pyridoxal hydrochloride (L. Light & Co.) were made up in 20% (v/v) ethanol at concentrations of 100 μg. free base/ml. and stored in a refrigerator.

The standard solutions used for addition to the assay tubes were prepared, by appropriate dilution, from these stock solutions.

Microbiological methods

Assays with Saccharomyces carlsbergensis 4228. The method was based on that of Atkin, Schultz, Williams & Frey (6). The test solutions, 0·5, 1·0, 2·0 or 4·0 ml., with duplicates at each level, were placed in optically matched Pyrex test tubes 19 × 150 mm. and the final volume was made to 4 ml. with distilled water. Standard solutions of pyridoxine or pyridoxal at a concentration of 3 mμg./ml. or pyridoxamine at a concentration of 5 mμg./ml. were treated in a similar way. The tubes were covered with aluminium caps (Oxo Ltd., London) and sterilized by heating in steam for 30 min. The double-strength assay medium, of composition shown in Table 1, was sterilized separately, cooled and added aseptically in 4 ml. quantities to each assay tube by means of an automatic syringe.

Table 1. *Composition per l. of the basal medium for assay with* Saccharomyces carlsbergensis 4228

Acid-hydrolysed casein (Allen and Hanbury Ltd.)	8·0 g.	Nicotinic acid	5000 μg.
		Pantothenic acid	5000 μg.
Citric acid	8·5 g.	Thiamine	500 μg.
Diammonium hydrogen phosphate	8·0 g.	Biotin	16 μg.
		pH	4·6
Sugar and salts solution*	500 ml.		
i-Inositol	50 mg.		

* Prepared as described by Atkin *et al.* (6)

The test organism, *S. carlsbergensis* 4228, was maintained on malt-wort agar slopes, transferred at monthly intervals and stored in a refrigerator. The inoculum was prepared by transferring a loopful of the yeast from a malt-wort slope, into 10 ml. basal assay medium supplemented with 10 mμg. each of pyridoxine, pyridoxal and pyridoxamine. The culture was incubated at 30° C. for 6 hr. with shaking, centrifuged and resuspended in basal medium containing no vitamin B_6. A further tenfold dilution in 0·9% (w/v) NaCl solution was made and one drop of this suspension used to inoculate each tube. Incubation was for 18–21 hr. at 30° C. with shaking. The assay tubes were then heated in steam for 10 min., cooled and growth was measured turbidimetrically in a Lumetron Model 400–A colorimeter.

Assays with Streptococcus faecalis *R ATCC* 8043. The double-strength basal medium was made as described by Rabinowitz & Snell (7) but with the following added/l. of medium: 10 mg. each of adenine, guanine and uracil; 2 ml. Tween 80; 800 μg. folic acid; and 200 mg. each of proline, histidine, phenylalanine and tyrosine. It was found necessary to treat the acid-hydrolysed casein (Allen and Hanbury Ltd.) with charcoal to reduce the medium 'blank', which was due to the presence of alanine. Tests with *Str. faecalis* were set up essentially as described for *S. carlsbergensis*, except that the total volume in the tubes was 10 ml., composed of 5 ml. test preparation or standard vitamin solution plus water, sterilized in the assay tubes, and 5 ml. double strength basal medium added aseptically after sterilization. The standard solutions of pyridoxal or pyridoxamine were made to a concentration of 1 mμg./ml. so that the effective assay range was from 0·5 to 4·0 mμg. pyridoxal or pyridoxamine per tube.

The assay organism, *Str. faecalis*, was maintained as a stab culture in yeast–liver-tryptone chalk agar, transferred at monthly intervals and stored at 4° C. The inoculum was prepared by growing the culture for 24 hr. at 30° C. in 10 ml. basal assay medium containing 10 mμg. pyridoxamine. The culture was then centrifuged, resuspended in 10 ml. 0·9% (w/v) NaCl solution and diluted a further ten times with saline. One drop of the suspension was used to inoculate each assay tube. Incubation was for 18 hr. at 30° C.

Assays with Streptococcus faecium Ø51. The culture was obtained from the Biochemical Institute, University of Copenhagen. It was grown in the same medium as described for *Str. faecalis*, except that charcoal treatment of the acid-hydrolysed casein was unnecessary and growth was improved by the addition of 1 g. DL-alanine/l. double-strength medium. The other assay conditions were the same as for *Str. faecalis*.

Assays with Lactobacillus casei *ATCC* 7469. The medium of Roberts & Snell(8) was used with vitamin B_6 omitted and 1 mg. riboflavin added/l. double-strength medium. The test conditions were similar to those described for *Str. faecalis*, the pyridoxal standard being used at the same concentration in both assays. *Lactobacillus casei* was maintained as a stab culture in the same manner as *Str. faecalis*. The inoculum was prepared by growing the culture for 7 hr. at 37° C. in 10 ml. basal assay medium containing 100 mμg. pyridoxal. This high level was necessary since a considerable proportion of pyridoxal was converted to pyridoxamine when the tubes containing the inoculum medium were sterilized by autoclaving. The culture was centrifuged, resuspended in saline and then diluted 1:100 in saline, and one drop of the suspension used to inoculate each assay tube.

Measurement of the pyridoxine, pyridoxamine and pyridoxal content of milk

The differential assay method of Rabinowitz & Snell(2) was used. The milk samples were prepared for assay by heating in steam for 30 min. in a large volume of dilute HCl as described above. The extracts so prepared were assayed simultaneously by the four test organisms *S. carlsbergensis*, *Str. faecalis*, *Str. faecium* and *L. casei*. The vitamin B_6 activities were calculated in terms of each of the analogues that were active for the test organisms. The method of calculating the pyridoxine, pyridoxamine and pyridoxal contents of the samples was as described by Rabinowitz & Snell(2).

Measurement of the effect of sterilization on the vitamin B_6 content of liquid whole milk

One litre of fresh milk was divided into five equal portions. One served as the control and was not heated. The other four were sterilized by heating at 110° C. for 30 min., one without any addition, one with pyridoxine hydrochloride, another with pyridoxamine dihydrochloride and the fourth with pyridoxal hydrochloride, all three analogues being added to give final concentrations of 0·3 μg. free base/ml. milk. The sterilized milk samples and the unheated milk were then assayed with the three test organisms (*S. carlsbergensis*, *Str. faecium* and *L. casei*) and their pyridoxine, pyridoxamine and pyridoxal contents calculated by the methods referred to above.

RESULTS

Activities of vitamin B_6 analogues for the different assay organisms

The relative activities of pyridoxine, pyridoxamine and pyridoxal for *S. carlsbergensis*, *Str. faecalis*, *Str. faecium* and *L. casei* are given in Table 2. Pyridoxal was about equally

as active as pyridoxamine for *Str. faecalis* and *Str. faecium* and gave a similar growth-response curve. With *S. carlsbergensis*, however, pyridoxamine was less active than the other analogues and the growth-response curve was of a different shape (Fig. 1).

Table 2. *Relative activities of pyridoxine, pyridoxamine and pyridoxal for the different test organisms*

(All results are calculated on the free-base content; the number of assays is given in parentheses.)

	S. carlsbergensis	*Str. faecalis R*	*Str. faecium* Ø51	*L. casei*
Pyridoxine hydrochloride	100	Inactive	Inactive	Inactive
Pyridoxamine dihydrochloride	63±12(22)	100	100	Inactive
Pyridoxal hydrochloride	106+ 9(22)	92±21(18)	87±12(12)	100

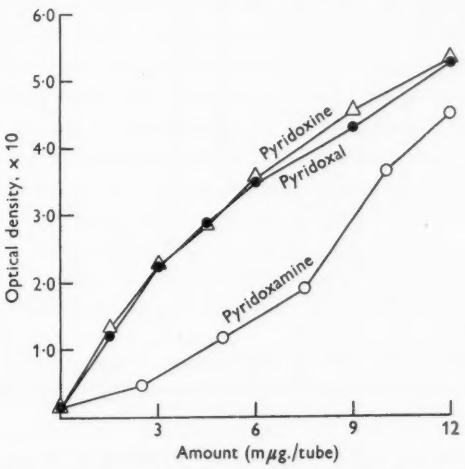


Fig. 1. Growth response of *S. carlsbergensis* to pyridoxine, pyridoxal and pyridoxamine

Effect of alanine on the growth of the different assay organisms

The addition of 5 mg. DL-alanine per tube caused no stimulation or inhibition of the growth of *S. carlsbergensis* in the absence or presence of pyridoxal. With *Str. faecalis*, the presence of 1 mg. DL-alanine per tube (i.e. per 10 ml. medium) resulted in almost maximal growth, even in the absence of pyridoxal or pyridoxamine and with glycine present in the medium (Fig. 2). On the other hand, DL-alanine could not replace pyridoxal or pyridoxamine as a growth factor for *Str. faecium*, although in their presence it was stimulatory (Fig. 3). For this reason, DL-alanine was added to the *Str. faecium* basal assay medium so that any alanine present in the test extract had no further stimulatory effect.

DL-Alanine replaced pyridoxal as a growth factor for *L. casei*, and stimulated growth in the presence of pyridoxal. 50 μg. DL-alanine per tube gave about half maximal growth in the absence of pyridoxal. L-Alanine, however, did not promote growth of *L. casei*, nor, in amounts up to 5 mg. per tube, did it stimulate or inhibit growth in the presence of pyridoxal. These results therefore confirm the earlier finding of Snell (9) that only the D isomer can replace pyridoxal in the metabolism of *L. casei*, whereas with *Str. faecalis* both D- or L-alanine are active.

Extraction of the vitamin B_6 activity from milk

In this work, in which the main object was to measure the effect of heat on the vitamin B_6 activity of milk, it seemed advisable to subject the samples to as little further heating as possible. Therefore, different methods of preparing the samples for assay were investigated. The results are presented in Table 3. Heating in steam with sodium acetate buffer

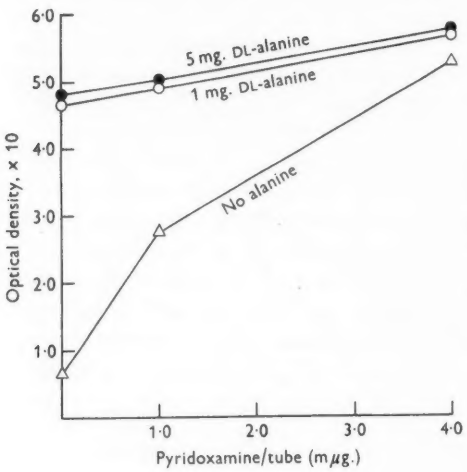


Fig. 2

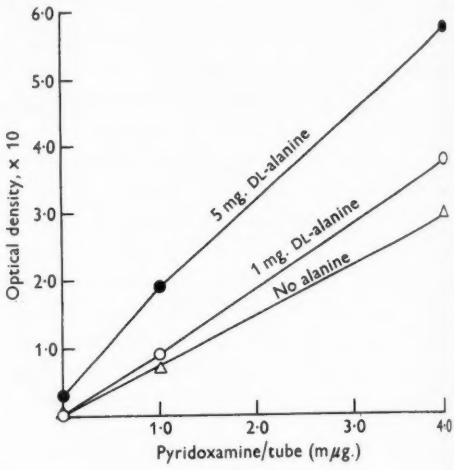


Fig. 3

Fig. 2. Effect of DL-alanine and pyridoxamine on the growth of *Str. faecalis*
Fig. 3. Effect of DL-alanine and pyridoxamine on the growth of *Str. faecium*

Table 3. *Comparison of different methods of extraction of the vitamin B_6 activity from milk, expressed as pyridoxal*

(Test organism: *Sacch. carlsbergensis*; conditions of assay as described in the text.)

Treatment	Raw whole milk (μg./ml.)	National dried milk (μg./g.)
Steamed 30 min. with sodium acetate buffer	0·33	2·00
Steamed 30 min. with 0·055 N-HCl	0·35	2·75
Autoclaved 30 min. at 120° C. with 0·055 N-HCl	—	3·00
Autoclaved 1 hr. at 120° C. with 0·055 N-HCl	—	2·73
Autoclaved 2 hr. at 120° C. with 0·055 N-HCl	—	2·64
Autoclaved 4 hr. at 120° C. with 0·055 N-HCl	0·39	2·98
Ultrafiltered	0·25	—

—, not tested.

for 30 min. resulted in a lower value than heating at 120° C. for 4 hr. in a large volume of dilute HCl. The vitamin B_6 activity of the milk treated with sodium acetate was not significantly altered by treatment with papain (British Drug Houses Ltd.), Takadiastase

(Parke, Davis & Co. Ltd.), pancreatin (British Drug Houses Ltd.), intestinal phosphatase (Armour Laboratories) or mylase P (British Drug Houses Ltd.). Since about 60% of the vitamin B_6 activity of raw whole milk was ultrafiltrable, further tests were made to discover whether the 4 hr. treatment was necessary for maximum release of the vitamin from a sample of National Dried Milk. The results in Table 3 show that heating the milk in steam for 30 min. in a large volume of acid gave results similar to those obtained after the milk had been heated at 120° C. for periods of up to 4 hr. Again treatment with sodium acetate buffer gave a lower value. These findings have been confirmed in tests with the other assay organisms and with raw whole milk. Therefore the procedure of heating the milk samples in steam for 30 min. in a large volume of dilute HCl has been used in all subsequent tests described in this paper.

The pyridoxine, pyridoxamine and pyridoxal contents of freeze-dried raw and evaporated milk and of commercial preparations of evaporated and condensed milk

In Table 4 the mean values and standard errors for the vitamin B_6 activity of the freeze-dried raw milk and the fresh and stored evaporated milk prepared from the same bulk are given in terms of each of the analogues active for the test organisms. From these values the amount of each analogue present in the sample was calculated according to the method of Rabinowitz & Snell (2) and the results are presented in Table 5. Values for pyridoxamine and pyridoxine were calculated from both the *Str. faecalis* and *Str. faecium* assays. The *Str. faecalis* values for pyridoxamine were higher than those given by *Str. faecium*. The corresponding high negative values for pyridoxine suggest that these pyridoxamine values are too high and, since *Str. faecalis* has been shown in the absence of any vitamin B_6 to use D- or L-alanine as a substitute, it is apparent that the strain of *Str. faecalis* used in these studies is not specific for measuring vitamin B_6 activity. *Str. faecium* is preferable since it cannot substitute alanine for pyridoxal or pyridoxamine and the values obtained with this organism are more in agreement with those obtained with *S. carlsbergensis* and *L. casei*.

From the values obtained with *Str. faecium* given in Table 5 (columns A only), it is apparent that pyridoxal predominates in raw milk. In the preparation of evaporated milk 63% of the original vitamin B_6 activity was lost. The pyridoxal content fell to 15% of the value in raw milk. The small amount of pyridoxamine present in the raw milk remained in the evaporated milk. There was a further loss of about 30% of the residual vitamin B_6 activity after storage of the evaporated milk for 6 months at room temperature.

From similar tests, with *Str. faecium* instead of *Str. faecalis*, values for the pyridoxine, pyridoxamine and pyridoxal contents of human, sow's and goat's milk, and of commercial samples of evaporated and condensed milks, were calculated (see Table 6). The human milk was low in vitamin B_6 activity and therefore the results with it are not precise. With sow's milk and goat's milk most of the activity was due to pyridoxal. The commercial samples of evaporated milk showed the same distribution pattern for the analogues as the freeze-dried evaporated milk prepared under experimental conditions. Most of the activity was due to pyridoxamine. After storage for 2 years very little pyridoxal was detected in the samples but some pyridoxine appeared to be present. The same was also found with the freeze-dried stored evaporated milk, and indicates that further changes in the vitamin B_6 activity of evaporated milk occur during storage.

Table 4. *Mean values with their standard errors for vitamin B_6 activities of freeze-dried samples of raw, freshly evaporated and stored evaporated milks measured with* S. carlsbergensis, Str. faecalis *R.*, Str. faecium Ø51 *and* L. casei *and expressed as μg.-pyridoxine, pyridoxamine or pyridoxal per g.*

(The number of assays is shown in parentheses.)

	S. carlsbergensis		*Str. faecalis* R.		*Str. faecium* Ø51		*L. casei*
Type of milk	Pyridoxine or pyridoxal	Pyridoxamine	Pyridoxamine	Pyridoxal	Pyridoxamine	Pyridoxal	Pyridoxal
Raw milk	3·38 ± 0·44 (12)	6·58 ± 1·11 (12)	4·56 ± 0·92 (10)	5·18 ± 0·92 (10)	3·0 ± 0·50 (7)	4·01 ± 1·01 (7)	3·20 ± 0·34 (11)
Freshly evaporated	1·00 ± 0·17 (12)	1·90 ± 0·26 (12)	3·72 ± 0·89 (10)	4·46 ± 0·96 (10)	1·25 ± 0·14 (7)	1·78 ± 0·41 (7)	0·46 ± 0·29 (11)
Stored evaporated	0·63 ± 0·05 (5)	1·45 ± 0·10 (5)	2·08 (2)	3·00 (2)	0·64 ± 0·08 (5)	0·92 ± 0·26 (5)	0·05 ± 0·04 (5)

Table 5. *Pyridoxal, pyridoxamine and pyridoxine contents of freeze-dried samples of raw, freshly evaporated and stored evaporated milk*

(Calculated by the method of Rabinowitz & Snell (2) from the results in Table 4. A, values calculated from *Str. faecium* Ø51 results; B, values calculated from *Str. faecalis* R results.)

	Pyridoxine		Pyridoxamine		Pyridoxal
Type of milk	A (μg./g.)	B (μg./g.)	A (μg./g.)	B (μg./g.)	(μg./g.)
Raw	−0·13	−0·71	0·60	1·73	3·20
Freshly evaporated	0·05	−1·22	0·93	3·34	0·46
Stored evaporated	0·30	−0·31	0·65	2·05	0·05

Table 6. *Pyridoxine, pyridoxamine and pyridoxal content of commercial samples of evaporated and condensed milks and of milks of different species*

(Calculated by the method of Rabinowitz & Snell(2); values given are the means of three assays.)

Type of milk	Pyridoxine (μg./ml.)	Pyridoxamine (μg./ml.)	Pyridoxal (μg./ml.)
Human	0·0	0·004	0·004
Sow's	0·0	0·01	0·26
Goat's	0·0	0	0·48
Sterilized cow's milk: fresh	0·02	0·10	0·13
stored	0·0	0·05	0·02
Evaporated milk (fresh)	(μg./g.)	(μg./g.)	(μg./g.)
Brand A	0·04	0·18	0·08
Brand B	0·12	0·19	0·04
Evaporated milk (stored)			
Brand A	0·26	0·09	0·01
Brand E	0·28	0·11	0·01
Brand F	0·29	0·10	0·01
Brand G	0·35	0·08	0·01
Condensed milk (fresh)			
Brand C	0·01	0·22	0·45
Brand D	0·03	0·25	0·14

Effect of sterilization on the vitamin B_6 content of raw whole milk

The effect of sterilization on the natural vitamin B_6 activity of milk and on pyridoxine, pyridoxamine or pyridoxal added to milk is shown in Table 7. As with the freshly evaporated milk the main loss of vitamin B_6 activity on sterilization was in the pyridoxal content. Added pyridoxine and pyridoxamine were recovered to the extent of 113 and 90% after sterilization and were therefore resistant to the heat treatment. However, of the added pyridoxal, 40% was recovered in its original form and 40% was converted to pyridoxamine, the rest being destroyed.

Table 7. *Effect of sterilization on the pyridoxine, pyridoxamine and pyridoxal contents of liquid whole milk, measured by the method of Rabinowitz & Snell* (2)

(Values are the means of two assays.)

	Pyridoxine (μg./ml.)	Pyridoxamine (μg./ml.)	Pyridoxal (μg./ml.)
Before sterilization	0·05	0·04	0·45
After sterilization:			
Alone	0·03	0·09	0·17
with 0·30 μg. pyridoxine/ml.	0·37	0·10	0·17
with 0·30 μg. pyridoxamine/ml.	0·04	0·36	0·17
with 0·30 μg. pyridoxal/ml.	0·03	0·20	0·30

With a commercial sample of sterilized milk the results were similar to those obtained with the milk sterilized in the laboratory. After storage for $6\frac{1}{2}$ years a sample of sterilized milk had very little vitamin B_6 activity and, compared with fresh samples, the loss of activity was in the pyridoxal content (see Table 6).

Treatment of vitamin B_6 analogues and milk with nitrous acid and alkaline acetone

It was found by the differential assay technique with the pure compounds that, as would be expected, nitrous acid did not attack pyridoxal or pyridoxine, but that pyridoxamine was partly converted to pyridoxine and partly rendered microbiologically

inactive. Similar tests showed that alkali and acetone inactivate 70–80% of pyridoxal without affecting pyridoxamine and pyridoxine.

In the milk samples treated in the same manner alkali and acetone destroyed much of the activity of raw milk for *L. casei*, *Str. faecium* and *S. carlsbergensis*, whereas nitrous acid treatment lowered the activity of raw milk for these organisms by only about 10%. These findings are consistent with pyridoxal being the form of the vitamin predominating in the raw milk. The fresh evaporated milk treated with alkali and acetone had from 33 to 50% of the activity of the untreated fresh evaporated milk for each of three test organisms. Nitrous acid treatment did not affect the *L. casei* result, but reduced that with *Str. faecium* by 75% and that with *S. carlsbergensis* by 33%. From these results it seems that freshly evaporated milk contains about equal amounts of pyridoxal and pyridoxamine. However, with the stored evaporated milk, treatment with alkali and acetone resulted in loss of less than 10% of the vitamin B_6 activity measured by the three test organisms, which suggests that little pyridoxal was present. On the other hand, nitrous acid treatment had no effect on the *L. casei* result, but almost completely destroyed the activity of the milk for *Str. faecium* and lowered the *S. carlsbergensis* value by about 25%. The smaller loss of activity measured with *S. carlsbergensis* in the fresh and stored evaporated milks heated with nitrous acid could be due to some conversion of pyridoxamine to pyridoxine.

DISCUSSION

The ability of D- and L-alanine to replace pyridoxal or pyridoxamine as a growth factor for *Str. faecalis* throws doubt on any results obtained with this organism for foodstuffs rich in protein. Milk is such a foodstuff. For a reliable estimate of the pyridoxine, pyridoxamine and pyridoxal content of the various milk samples by the method of Rabinowitz & Snell(2) a more specific measurement of total pyridoxal plus pyridoxamine was required. This was achieved with the strain of *Str. faecium* recommended by Møller(10). This organism requires pyridoxal or pyridoxamine for growth but, unlike *Str. faecalis*, it cannot substitute alanine for them though alanine stimulates its growth in their presence. Inclusion of DL-alanine in the basal medium prevents stimulation of growth by alanine present in the test extracts and a reliable measure of total pyridoxal plus pyridoxamine can be obtained.

In a study of the forms of vitamin B_6 present in natural materials, one of the most important problems is that of extracting the vitamin from the test material without altering its form. The procedure recommended by Rabinowitz & Snell(11) for extracting vitamin B_6 active substances from natural materials is to autoclave the sample for 5 hr. at 120° C. in a large volume of dilute hydrochloric acid. Although they showed that this treatment did not destroy the pure vitamin B_6 analogues, it seemed drastic for milk in which, as shown by ultrafiltration experiments, 60% of the vitamin activity is already free. The results reported in this paper show that the vitamin B_6 active substances in milk can be extracted just as readily by heat treatment reduced from 5 hr. at 120° C. to 30 min. at 100° C. This procedure not only made it easier to set up the tests in 1 day but also reduced the risk of changes during prolonged heating of the samples.

Rabinowitz, Mondy & Snell(12) have shown that when pyridoxal is autoclaved at neutrality with glutamic acid, transamination occurs and pyridoxal is converted to pyridoxamine. At pH 1·8 transamination does not take place and therefore pyridoxal can be extracted unchanged from a test material containing amino groups if the pH of the

extractant is low. These conditions obtain when a large volume of 0·055 N-HCl is used. If, however, test extracts so prepared are then autoclaved with the basal assay medium, transamination could occur. In the experiments described in this paper, the basal media were sterilized separately and added aseptically to the sterile test extracts immediately before inoculation of the assay tubes with the test micro-organism. In this way the inactivation of pyridoxal was avoided. Since transamination between pyridoxal and amino acids is favoured by heat treatment, it would be expected that most of the pyridoxal of milk would be converted to pyridoxamine during the process of sterilization. However, the values for the pyridoxine, pyridoxamine and pyridoxal contents of raw and sterilized (both liquid and evaporated) milk reported in this paper show that this change does not happen to any great extent. Most of the pyridoxal activity naturally present is lost with only a small corresponding increase in the pyridoxamine content. Furthermore, when pyridoxal, pyridoxamine or pyridoxine were added to liquid whole milk before sterilization, the pyridoxamine and pyridoxine resisted the heat treatment, whereas pyridoxal was partly converted to pyridoxamine and partly destroyed.

Hassinen, Durbin & Bernhart (13) found that pyridoxine added to milk resists heating for 1 hr. at 121° C., but in their tests both pyridoxamine and pyridoxal were partly destroyed under these conditions which are more drastic than those used in our laboratory. They also reported a 50–60 % loss of vitamin B_6 activity during the preparation of evaporated milk, whereas Hodson (3) using the same assay organism (*S. carlsbergensis*) reported only a slight loss in freshly evaporated milk, but a more marked loss after storage of the milk for several months. In an earlier paper from this laboratory, Chapman, Ford, Kon, Thompson, Rowland, Crossley & Rothwell (14) reported no loss of vitamin B_6 activity in freshly evaporated milk. They suggested that other factors, such as the previous history of the milk in relation to aeration and exposure to light, oxygen in the head space of the container and the period and temperature of storage after sterilization, also determine the extent of the loss of vitamin B_6 activity. My results for evaporated milk indicate that the major loss of activity occurred within the 2 weeks of storage at 4° C. which elapsed between its preparation and the freeze-drying. Subsequent storage of the liquid evaporated milk at room temperature for 6 months resulted in a further loss of activity, whereas the freeze-dried evaporated milk stored at 4° C. in air-tight jars during this same period showed no such loss.

Hodson (3), using the methods of Snell (5) for destroying pyridoxal with alkaline acetone, and pyridoxamine with nitrous acid, concluded that stored evaporated milk contains very little pyridoxal and that most of the residual vitamin B_6 activity is due to something other than pyridoxal or pyridoxamine. The experiments described here in which similar tests were used agree with this finding. The differential assay method of Rabinowitz & Snell (2) indicates that the residual activity might be due to pyridoxine. However, Rabinowitz & Snell (2) have pointed out that their differential assay method has its limitations. One of them is that only the pyridoxal value can be considered as accurate since it is measured directly, whereas the other two analogues are measured by difference. Pyridoxine is the least accurately determined and therefore its presence in the stored evaporated milk cannot be established by this method. Further work is now in progress to isolate and identify this factor from evaporated milk.

The comparison of biological and microbiological assays of vitamin B_6 in raw and evaporated milk is presented in the accompanying paper (4).

SUMMARY

1. Four test organisms were used for the microbiological assay of vitamin B_6. The test organisms and the forms of vitamin B_6 active for them were: *Saccharomyces carlsbergensis* 4228 (pyridoxine, pyridoxamine and pyridoxal), *Streptococcus faecalis* R ATCC8043 and *Str. faecium* Ø51 (pyridoxamine and pyridoxal), and *Lactobacillus casei* ATCC7469 (pyridoxal).

2. By simultaneous assay of the test material with the four organisms the amounts of pyridoxine, pyridoxamine and pyridoxal present were determined. The differential assay technique has been improved by the use of *Str. faecium*, which is more specific for measuring total pyridoxamine plus pyridoxal, instead of *Str. faecalis*.

3. The optimum conditions for measuring vitamin B_6 in milk were studied and the differential assay technique was applied in an investigation of the effect of heat on the vitamin B_6 activity of milk.

4. It was found that pyridoxal accounted for about 80% of the vitamin B_6 activity of raw milk, the other 20% being due to pyridoxamine.

5. In the manufacture of evaporated milk there was a marked fall in the pyridoxal and a slight increase in the pyridoxamine content. The overall loss was some 60%.

6. On storage of evaporated and of sterilized liquid milk at room temperature there was a further loss of vitamin B_6 activity. Most of the residual activity in stored evaporated milk appeared to be due to a substance other than pyridoxal or pyridoxamine, possibly pyridoxine.

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THE EFFECT OF HEAT ON THE VITAMIN B_6 OF MILK

II. A COMPARISON OF BIOLOGICAL AND MICROBIOLOGICAL TESTS OF EVAPORATED MILK

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Unsweetened evaporated milk is widely used in infant feeding. This product is subjected to drastic heat treatment during sterilization in the can. Lately there have been several reports (1, 2, 3, 4, 5, 6) that in the process of manufacture evaporated milk loses a large part of its original vitamin B_6 activity. According to Tomarelli, Spence & Bernhart (5) the loss of vitamin B_6, as judged microbiologically, is much less than that determined with rats. We present here the results of a reinvestigation of the problem. The vitamin B_6 activities of raw, evaporated and stored evaporated milk were measured with rats, and chicks, and also microbiologically. In addition, the activities of the pure analogues for chicks and rats were determined to give a basis for comparison. The microbiological techniques have been separately studied by one of us and the findings are set out in the accompanying paper (7).

EXPERIMENTAL

Materials

From a 36 gal. bulk of fresh mixed full cream milk from the N.I.R.D. herd, 18 gal. were freeze-dried immediately. The other 18 gal. were evaporated, canned and sterilized as described in (8). Half was freeze-dried immediately and half after 6 months storage at room temperature. The freeze-dried milks were stored at $-15°$ C. in air-tight containers until required.

Solutions of the pure vitamins, pyridoxine hydrochloride and pyridoxamine dihydrochloride (Roche Products Ltd.) and pyridoxal hydrochloride (L. Light and Co.) were made to contain 500 μg. free base/ml. and used as described below.

Chick assays

The method developed by Coates, Kon & Shepheard (9) was used. The basal diet given in Table 1 contains, with the exception of vitamin B_6, all characterized vitamins believed to be essential for the chick. With vitamin B_6 added in sufficient quantities it supports excellent growth. As prepared, the diet is a fine powder not readily eaten by chicks and past experience has shown that it is much better granulated.

The freeze-dried milk samples were incorporated into the diet to form 25, 12·5 or 6·25% of it, replacing an equal weight of casein and dextrin (in the ratio 3:2). These quantities were too large to be given separately by mouth. For this reason the pure analogues were tested in the same way, being included during granulation at the rate of 250, 125, 62·5 or

31·25 μg. free base/100 g. diet. In addition, they were also tested separately, given by mouth three times weekly as 0·1 ml. of a solution containing 500, 250, 125 or 62·5 μg. free base/ml. For granulation, the diet was mixed in a Hobart dough mixer with 40% water containing, when appropriate, the pure vitamin B_6 supplements. The resulting mixture was rubbed through a ¼ in. mesh sieve, spread in ½ in. deep layers on trays and dried in a forced-draught oven at 40° C. The pyridoxine, pyridoxal and pyridoxamine content of the granulated diets was determined by the differential assay method of Rabinowitz & Snell (10) as described by Gregory (7).

Table 1. *Percentage composition of the vitamin B_6-deficient diet used in the chick assays*

Dextrin	60·45	L-Cystine	0·3
Casein (low vitamin, Genatosan Ltd)	18	Choline chloride	0·15
Gelatin	10	Inositol	0·10
Salt mixture*	6	Arachis oil†	5·0

* Percentage composition of salt mixture: $CaCO_3$ 25·0, K_2HPO_4 26·9, $CaHPO_4.2H_2O$ 22·9, NaCl 13·9, $MgSO_4.7H_2O$ 8·5, $Fe(C_6H_5O_7)_2$ 2·3, KI 0·067, $MnSO_4.4H_2O$ 0·42, $ZnCl_2$ 0·02, $CuSO_4.5H_2O$ 0·025.

† Containing in 5 g. 160 i.u. vitamin D_3, 1700 i.u. vitamin A as halibut liver oil concentrate, 0·5 mg. menapthone, 0·3 mg. α-tocopherol.

To every 100 g. of diet were added 0·02 mg. biotin, 0·075 mg. folic acid, 0·3 mg. thiamine hydrochloride, 0·6 mg. riboflavin, 1·5 mg. calcium pantothenate, 4 mg. nicotinic acid and 0·002 mg. vitamin B_{12}.

Day-old Rhode Island Red × Light Sussex cockerel chicks were used, 15–18 per group, housed in electrically-heated, tiered brooders with wire floors. They were supplied with food and water *ad lib.* In those groups receiving the lower levels of vitamin B_6 supplement, either as milk or as the pure analogues, convulsions typical of insufficiency of vitamin B_6 and some deaths occurred from the seventh day. For this reason assays were ended at about the twelfth day when only half the birds in such groups remained. The criterion of response was the final body weight.

Rat assays

Litter-mate male black-hooded rats from our colony were weaned on to the vitamin B_6-deficient diet shown in Table 2 and were depleted on it for 3 weeks. At the end of this period the animals were put into separate cages and one member of each litter was as-

Table 2. *Percentage composition of the vitamin B_6-deficient diet used in the rat assays*

Glucose	40	Arachis oil (semi-hardened)	12
Lactose	17	Salts (11)	4
Casein (low vitamin, Genatosan Ltd.)	25	Vitaminized arachis oil (12)	2

To every kg. of diet were added 220 mg. inositol, 100 mg. nicotinic acid, 100 mg. Ca-pantothenate, 75 mg. *p*-aminobenzoic acid, 30 mg. thiamine, 30 mg. riboflavin, 0·2 mg. biotin, 1 mg. folic acid, 300 mg. choline chloride and 20 μg vitamin B_{12}.

signed at random to each level of standard or test material. The number of rats in separate assays varied between 9 and 11 in each group. The rats received the unsupplemented diet *ad lib.* The supplements, whether milk or vitamin solutions, were pipetted into special feeding dishes daily, except on Saturdays when a double dose was given to cover Sunday. The solutions of the vitamin B_6 analogues were diluted so that the concentration was

10 μg./ml.; daily doses of 2 or 4 μg. were given. The freeze-dried milks were dispersed in water so that estimated doses of 2 or 4 μg. were contained in 5 or 10 ml. of reconstituted milk. All vitamin additions were completely consumed within a few minutes. The criterion of response was the gain in weight during a 4-week experimental period.

Statistical analysis

The values obtained in rat and chick assays were analysed by the procedure given in B.S. 911 (13). The variance due to differences between litters was subtracted from the total error variance in the rat assays. True fiducial limits were calculated for individual assays, and approximate limits of error for the pooled values of all assays.

RESULTS AND DISCUSSION

Activity of pyridoxine, pyridoxal and pyridoxamine for rats and chicks

The relative potencies of the three forms of vitamin B_6 are given in Table 3. When given separately from the diet the three analogues were equally active for both species, in agreement with the findings of others (14, 15). When included in the diet pyridoxamine

Table 3. *Activities of vitamin B_6 analogues for chicks and rats (pyridoxine = 100%)*

Given separately from diet	Test 1	Test 2	Test 3	Pooled
Rats				
Pyridoxal	111 (93–132)	105 (86–130)	—	108 (96–122)
Pyridoxamine	82 (68–97)	—	90 (73–111)	85 (75–97)
Chicks				
Pyridoxal	102 (71–149)	85 (64–113)	92 (77–110)	93 (80–105)
Pyridoxamine	—	99 (77–128)	96 (81–114)	97 (85–112)
Given in the diet				
Chicks				
Pyridoxal	56* (43–72)	83* (68–100)	71* (61–82)	70 (45–113)
Pyridoxamine	81 (67–99)	85 (69–105)	80 (73–88)	81 (75–88)

Values in parentheses are true fiducial limits for the individual tests and approximate limits of error for the pooled values.
* Values significantly different from each other ($0{\cdot}05 > P > 0{\cdot}01$).

was in three tests with chicks about 80% as active as pyridoxine. The microbiological assay of the diets (Table 4) shows that the pyridoxamine diets as prepared contained only about 80% of the expected activity and that there was no loss during the test period. It is reasonable to assume that the lowered activity of pyridoxamine was due to its destruction during the preparation of the diet. When mixed in the diets pyridoxal was also much less active for chicks than pyridoxine, but the degree of inactivation varied markedly in the three tests. The results of the microbiological tests of the chick diets were also divergent both in total recovery and in the distribution of the analogues: in test 2 all the vitamin was recovered as pyridoxal, whereas in the other two tests 2–13 and 3–20%, respectively, of the original pyridoxal was converted into pyridoxine and pyridoxamine. The microbiological assays gave a higher total value than the chick assay. It

is possible that pyridoxal forms complexes with amino acids in the diet which are as unavailable to the chick as to the rat (16), but which are reconverted to the free form during the preparation of the extract for microbiological assay.

Table 4. *Vitamin B_6 content of chick diets*

	Microbiological assay,* percentage recovery					
				Total value of vitamin B_6		
Analogue added to diet	Pyridoxine	Pyridoxal	Pyri-doxamine	Value	Expressed as percentage of value for pyridoxine†	Chick assay expressed as percentage of value for pyridoxine
Test 1						
Pyridoxine *a*	99	0	1	100	100	100
b	130	0	0	130	100	—
Pyridoxal *a*	2	87	20	109	109	56
b	11	92	3	106	81	—
Pyridoxamine *a*	0	4	105	109	109	81
b	3	5	105	113	87	—
Test 2						
Pyridoxine *a*	96	0	0	96	100	100
Pyridoxal *a*	0	90	0	90	94	83
Pyridoxamine *a*	0	6	78	84	87	85
Test 3						
Pyridoxine *a*	96	0	0	96	100	100
Pyridoxal *a*	13	55	16	84	87	71
Pyridoxamine *a*	0	5	72	77	80	80

* By the differential assay method of Rabinowitz & Snell (10) with *Saccharomyces carlsbergensis*, *Streptococcus faecalis* and *Lactobacillus casei*.
a Samples taken at beginning of chick test.
b Samples taken at end of chick test.
† Comparisons made between samples taken at the same time.

Sarma, Snell & Elvehjem (15) found some loss of activity of both pyridoxamine and pyridoxal when given to chicks in the diet, but later Waibel, Cravens & Snell (17) reported that inactivation occurred when the carbohydrate in the diet was soluble (glucose), but not when it was insoluble (dextrin). They suggested that the soluble-carbohydrate diet encouraged the growth of lactic acid bacteria which preferentially absorbed the vitamin, thus removing some of it from the economy of the chick. However, Luckey, Briggs, Elvehjem & Hart (18) using dextrin also found a loss. In our experiments, but not in those of other authors (15, 17, 18), the diet was granulated. The loss of activity of pyridoxamine and of part of that of pyridoxal was probably due to a true destruction during the making of the diet, perhaps during the granulation process. It is possible that such a destruction could also occur in an ungranulated diet, possibly in the presence of casein, which could explain the divergent results of Luckey *et al.* (18) and of Waibel *et al.* (17). However, these workers did not check microbiologically the vitamin B_6 content of their diets.

The vitamin B_6 activity of milks

The vitamin B_6 activities of freeze-dried preparations of raw, evaporated and stored evaporated milk for rats, chicks and *Saccharomyces carlsbergensis* are given in Table 5. The results for raw milk by the three methods are in fair agreement in view of the errors of microbiological and biological tests and of the need to express the vitamin B_6 activity in terms of one of the analogues only (in these tests, as pyridoxine).

For the evaporated milk, whether fresh or stored, the biological values are higher

than the microbiological one. We have at present no explanation to offer for this discrepancy.

Table 5. *Vitamin B_6 content of freeze-dried samples of raw, evaporated and stored evaporated full cream milk (measured as μg. pyridoxine/g.)*

		Test method						
		Chick			Rat			
Type of milk	*S. carlsbergensis**	Test 1†	Test 2†	Pooled‡ value	Test 1†	Test 2†	Test 3†	Pooled‡ value
Raw	3·38±0·44	2·8 2·1–3·6	3·4 2·8–4·0	3·2 2·8–3·7	6·0 4·9–7·5	4·5 3·9–5·1	5·1 4·1–6·4	4·9 3·7–6·7
Evaporated	1·00±0·17	2·1 1·5–2·8	2·1 1·7–2·4	2·1 1·8–2·4	2·6 2·1–3·2	2·8 2·4–3·2	2·8 2·3–3·5	2·7 2·0–3·7
Stored evaporated	0·63±0·05	—	1·4 1·2–1·7	—	—	—	2·0 1·6–2·5	—

* Value with its standard error.
† Value with true fiducial limits.
‡ Value with approximate limits of error.

The fact remains that measurements by three methods show a loss of some 45–70% of the total vitamin B_6 activity during the preparation of evaporated milk, and a further loss of some 30% of the remainder during storage for 6 months at room temperature. Our results agree with these of Tomarelli *et al.* (5) in that both microbiological and biological assays show a loss of vitamin B_6 activity in evaporated milk. We could not confirm their conclusion that the activity for the rat is less than that for *S. carlsbergensis*: on the contrary we found the reverse.

Our microbiological and biological tests reported here and in (7) show that the loss of vitamin B_6 activity occurring during the manufacture of evaporated milk cannot be entirely accounted for by a change to some undefined, less active form of the vitamin but is most probably a true destruction.

Tests with *S. carlsbergensis* are simpler and speedier than biological assays. In addition, *S. carlsbergensis* tests show a greater loss in vitamin B_6 activity of milk after heat-treatment during evaporation. In our opinion, therefore, they can be used with confidence to provide the upper limit of damage to biological activity and serve as a useful guide to the adequacy of the vitamin B_6 content of evaporated milk products in infant foods.

SUMMARY

1. For chicks and rats pyridoxine, pyridoxal and pyridoxamine were equally active in terms of the free bases when given separately from the diet.

2. Under our experimental conditions pyridoxine mixed with the chick diet was stable, but 20% of pyridoxamine, and a variable amount of pyridoxal was lost.

3. The vitamin B_6 activities measured with *Saccharomyces carlsbergensis*, chicks and rats respectively and expressed as μg. pyridoxine/g. freeze-dried milk were: raw milk 3·4, 3·2 and 4·9; evaporated milk 1·0, 2·1 and 2·7; stored evaporated milk 0·6, 1·4 and 2·0. For the chicks the milks were mixed with the diets; they were given separately to the rats.

4. The microbiological and biological results for raw milk agreed within the limits of experimental error. For the processed milks the differences between biological and microbiological tests were statistically significant.

5. All three methods of assay showed a 45–70% loss of vitamin B_6 activity on processing and a further loss of 30% of the remainder after storage for 6 months at room temperature.

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